Universidad Autónoma de Madrid

Departamento de Bioquímica

Identificación de nuevas señales extracelulares que median sus actividades biológicas vía Cot/tpl-2

Carlos Sanz García

Madrid, 2014

Departamento de Bioquímica

Facultad de Medicina

Universidad Autónoma de Madrid

Identificación de nuevas señales extracelulares que median sus actividades biológicas vía Cot/tpl-2.

Memoria de Tesis Doctoral presentada por:

Carlos Sanz García

Licenciado en Bioquímica, para optar al grado de Doctor

por la Universidad de Madrid

Directora de la Tesis:

Dra. Susana Alemany de la Peña

Investigadora científica

Instituto de Investigaciones biomédicas “Alberto Sols”

CSIC-UAM

Dra. Susana Alemany de la Peña, Investigadora Científica del Instituto de Investigaciones Biomédicas "Alberto Sols" CSIC-UAM, certifica que:

Carlos Sanz García, Licenciado en Bioquímica por la Universidad de Valencia, ha realizado bajo mi dirección el trabajo de investigación titulado: "**Identificación de nuevas señales extracelulares que median sus actividades biológicas vía Cot/tpl-2.",** en el Instituto de Investigaciones Biomédicas "Alberto Sols".

Considero que el mencionado trabajo es satisfactorio y apto para poder optar al grado de doctor por la Universidad de Madrid.

Y para que conste a todos los efectos, firmo el presente certificado en Madrid, a 07/01/14.

Fdo. Susana Alemany de la Peña

Directora de la Tesis

Investigadora Científica

V°B° Tutor/a

Lisardo Boscá Gomar

Profesor Investigador

Este trabajo ha sido realizado en el Departamento de Bioquímica del Instituto de Investigaciones Biomédicas “Alberto Sols” (UAM-CSIC), gracias a una Ayuda a la Investigación para Personal Investigador en Formación del Ministerio de Economía y Competitividad.

A mis padres

A mi hermano

Para ser irremplazable, uno siempre debe ser diferente.

-Coco Chanel

Agradecimientos

Todo momento llega en la vida y cuando empiezas un camino como este, nunca eres capaz de ver la luz al final del túnel. Pero existe, y un día cuando menos te lo esperas empiezas a verla, con las penumbras que eso supone hasta llegar a una libertad total. Por ello me gustaría recordar cada uno de esos pasos que he dado, y a las personas que me han visto y ayudado a darlos.

Primeramente, a mi tutora de Tesis, la Dra Susana Alemany por su confianza puesta en mí. Ella creyó en un valenciano que estaba dispuesto a todo, dejando atrás su vida para empezar una nueva etapa en la gran ciudad. Ha habido momentos para todo, risas, lloros, desilusiones, alegrías, etc, pero el resultado ha sido muy satisfactorio, mucho más de lo que yo esperaba. Así que muchísimas gracias por tu tiempo, tu esfuerzo y tu dedicación, y sobre todo por darme la oportunidad de emprender este viaje en el mundo de la investigación. También agradecer a la Dra Margarita Fernández por toda su ayuda. Sin ti y tus chicas, parte de este camino hubiera sido mucho más lento y tortuoso. Y sobre todo por todas las palabras de ánimo, explicaciones de cualquier tema y protocolo, y demás conversaciones.

El camino empezó en Santander, bonita ciudad donde estuve aprendiendo como manejar esos bichitos con los que luego iba a trabajar. Mai fuiste una profe excelente y muchas veces me acuerdo de ti y de tu chico Val, y de todos los consejos que me diste. Recordar a Marcos y a Jorge, que aun os estoy esperando en Madrid para liarla.

Tras unos meses duros de trabajo con lluvia y más lluvia, llegue a Madrid para conocer el B-15 en todas sus dimensiones. El comienzo no fue nada fácil, acostumbrarse a donde están las cosas y como se hacen, animales, servicios, gente. Pero poco a poco fui conociendo más, y Roberto me enseño mucho de cómo tratar y manipular nuestros animales, además de tener siempre la disposición de ayudarte en cualquier momento para cualquier cosa. Al mismo tiempo que yo llegue, una gran persona se marchó del laboratorio dejándome su sitio, desde donde hoy escribo estas palabras. Irene Soria Castro, viví poco en el labo contigo, pero lo que he vivido fuera me ha servido para darme cuenta de la maravillosa persona y amiga que eres. Momentos legendarios que jamás seremos capaces de olvidar, bodas (coño, tu boda), navidades, borracheras, tesis… Muchas gracias por todo, de verdad. Noelia y Gemma, estudiantes de la avanzada que me enseñaban más a mí que yo a ellas. Pero de aquí guardo un gran recuerdo con Gemma, vivimos un par de añitos juntos y finalmente pudiste entrar a hacer la tesis con Alberto. Cuando me dijiste que al final te había dicho que sí, pensé: y a quien le va a preguntar ahora todo? Y mírate, no paras de hacer cosas, eres autosuficiente en el labo y además con tu estancia en los EEUU. No veas las vueltas que da la vida. Al tiempo llego Clara, otra estudiante de avanzada que fue de gran ayuda para mí. Con ella me di cuenta que aunque parezca que no, el llevar en un tiempo en un lugar te hace estar mucho más desenvuelto y poder resolver los problemas y conflictos con mayor rapidez. No quiero dejarme a esos niños y niñas que

vinieron para hacer las prácticas de su módulo y que tanto trabajo nos evitaron. A Raquel, que era una pizpireta y no paraba, joer que marcha tenía; Alejandro y Ángela, que parecían Zipi y Zape los dos juntos para todos lados; y por su puesto a mis niñas Amanda y Liset, que aunque me ha costado al final he hecho de vosotras grandes mujeres (tono irónico). Gracias por esos contajes de células interminables, sin vosotras me hubiera dejado más los ojos. A Marta, has sido un gran pilar para mí. En todo este tiempo no solo has sido mi compañera, sino que me has enseñado todo en el labo. Sé que muchas veces era muy pesado, pero para hacer las cosas como la mejor, debes aprenderlo todo como la mejor. Solo deseo que tu etapa fuera de España vaya ni la mitad de bien que te fue aquí, sobretodo porque a trabajadora y luchadora no te gana nadie. ¡Hija, deja algo pa los demás que en España con la crisis están las cosas muy mal! Y por último, acordarme de Nacho otro valenciano con el que llenamos el labo de risas. Fijo que estamos un tiempo trabajando juntos, así que mucho ánimo y a darlo todo. Eso sí, corre con tu tesis que te pillo! Al final no, te me adelantaste!!!!

Cambiando de laboratorio y de lugar, nos vemos al 1.3.1. Pepe y las Marías. No soy capaz de tener un recuerdo bonito de vosotros sin que aparezca otro por ahí. Grandes momentos en comidas, meriendas y cenas, y bodas, y navidades, y viajes… Cuantas cosas hemos pasado juntos! Sin duda, las mejores bodas para mí han sido con todos vosotros. La de Pepe en Alicante, con ese viaje a la playa en pleno agosto, y la de Irene en Madrid, un poco más cerquita, pero casi con el mismo calor. Pepe, el súper papi de Gabriel, ahora toca vivir otra etapa, así que disfruta del pequeñín porque luego crecen y te la lían. Pacheco, grande en todo, desde hacer videos hasta bailar y coser. Eso sí, nos debemos un parque de atracciones en condiciones a lo grande para disfrutar a saco, porque si yo soy un niño, tú y Valencia estáis a mi nivel. Y ahora con una ratita en camino… Enhorabuena! Y Valencia, mi Manolo preferido, no veas como conduce. En otra época, Alonso no sería nada a su lado. Pero hemos tenido el gusto de tenerla entre nosotros, ayudando, luchando, chillando, vamos de todo, un no parar. Eso sí, deja de copiarme las frases que sé que te vuelven loca. ¡Ánimo!

Mi pasillo en el departamento también me hace recordar a muchísima gente con la que he compartido horas en células y servicios. A todos y cada uno de los laboratorios. Nombrar a todas las personas es muy difícil porque siempre alguien te ha ayudado, o te ha dado un consejo, pero quiero acordarme especialmente de aquellos que han estado o están en mi misma situación. Del B-20, mi Leti. Mucho ánimo y mucha fuerza. Estaremos en contacto porque nos tenemos que ver en California, no? Del B-19, a la franchute de Luci, y a la gallegiña que me alegraba todos los días, y yo a ella por su puesto, Merce. Del B-16, al italiano que me enseñaba las canciones de Raffaella, Giacomo y a Fer, otro valenciano postizo, si es que la tierra se nota. Del B-11, a mi Pimentel que hemos estado a la par en casi todo, hasta a la hora de escribir la Tesis. Hija no me voy a deshacer de ti ni para eso!!

A Laura, la persona más eficiente que jamás nadie se podrá encontrar en citometría, que pase lo que pase, siempre te está para ayudarte en todo. A Lola y Anuska, que aunque no os haya dado mucho la tabarra, me he pasado más a cotillear y a no dejaros trabajar. Ana, muchas gracias por engancharme a todas esas series, no veas!!! No tenía tiempo de nada, solo de verlas y pedirte nuevos capítulos. Y yo que pensaba que EEUU vería todo antes que tú, y al final me tenías que subir tú los vídeos. Qué triste, como me vaya a cruzar el charco te aseguro que veré los capítulos antes que tú y te los contare. Del IIB me acordaré de Genómica, y sus PCRs; Imagen, con Antonio y Javi y todas las imágenes, tablas, gráficas y figuras; Animalario, Por la ayuda brindada en todo momento; y de Confocal, con súper Diego explicándome mil veces como enchufar el microscopio.

Mención especial se merece María Galardi. Una de las personas más atareadas del mundo mundial. Que intentas quedar con ella, avisa con tiempo porque va a ser imposible. Entre patines, padel, joyería, solo te falta coser y montarte una tienda. Eso sí, si lo haces acuérdate de María valencia y de mí, que tenemos máquina, aunque María no sepa enhebrar la aguja todavía. Dale su tiempo! Pues eso, que ha sido un placer enorme conocerte y compartir contigo viajes, bodorrios y demás. Y Por otro lado Isa, y todos sus xicos. Tus risas, chistes y viajes, hacían que las comidas fueran mucho menos aburridas porque siempre había algo de lo que hablar…

Y por último, acordarme de toda la gente de Cleveland, que me acogió y ayudó como uno más. A la Dra Laura Nagy por darme la oportunidad de realizar una estancia en su laboratorio y por toda su enseñanza. A cada una de las personas que allí trabajan pero sobre todo a Megan y Dola, my girls. Muchos momentos vividos juntos, cenas, compras, bailes… Creo que han sido unas de las mejores Navidades de mi vida, sin tener la familia cerca y eso era porque me hicisteis sentir como parte de ella. Recuerdos de América los tendré siempre conmigo, y es porque empezó como una experiencia profesional con el mundo solo para mí, y acabaron siendo las navidades más blancas (porque hubo mucha nieve), con mi familia en NYC. Awesome!!!!

De esta experiencia me voy a llevar muchas cosas y muchas personas, pero como Gustavo y Rubén, nadie. Mis malagueños! Con vosotros he viajado, he reído, he llorado, y siempre he sentido que aunque mi gente estuviera muy lejos, os tenía cerquita como parte de vosotros. No soy capaz de separaros porque sois muy importantes para mí. Grandes personas y grandes amigos, este donde este y pase lo que pase, esta época siempre estará conmigo y vosotros también.

Hay mucha gente que recordar y que no han estado aquí para darme su apoyo pero sí que lo hacían desde la distancia. En valencia hay mucha gente que en algún momento de su vida ha pensado: ¿Dónde parará Carlos, o que estará haciendo? A todas ellas muchas gracias por parar un momento y recordarme como yo lo hago continuamente. A mi falla, Mariano Benlliure-

Acequia de tormos que cada vez que vuelvo, no me dejan ni cenar de los besos y abrazos que tengo que dar. Y a mi querido Forcat, volver es sentirme como el hijo prodigo. Siempre que bailo, me hacéis sentir especial y eso es porque lo sois, todos y cada uno de vosotros. A Álvaro y a Sara, mis dos grandes apoyos en casa. Sea como fuere, digo que voy un finde y paráis el mundo por verme, estar conmigo y pasar unas risas o fiestas juntos. Sois parte de mi vida, ahora y siempre. Gracias!!!

Y solo me falta agradecer a las personas más especiales del mundo que conozco, mi familia. Papá y tete, Pedro e Iván, según hemos ido creciendo, y después de nuestra perdida, hemos ido consolidándonos como una familia muy fuerte. No sé si muchos de los momentos que he vivido aquí hubieran sido posibles sin saber que estabais ahí apoyándome. Muchas gracias por todo, por ser parte de mí y por quererme tal como soy.

Creo que si tuvieras la oportunidad de estar aquí, serías la persona más feliz del mundo, viendo a tu hijo acabar una etapa por la que ha luchado durante mucho tiempo. Siempre estuviste conmigo en todo, y más que nunca quiero recordarte para siempre, como lo hago cada día de mi vida. MAMÁ, te quiero!

Resumen / Abstract

La familia de receptores TLR son sensores de las infecciones de patógenos por su capacidad de reconocer y activarse en respuesta a patrones moleculares que se encuentran conservados en dichos patógenos (PAMPs). En los últimos años se ha demostrado que los TLRs así como otros receptores incluidos en la superfamilia de receptores que reconocen patrones moleculares (PRRs) también reconocen moléculas propias endógenas del organismo que en condiciones fisiológicas no están físicamente en el mismo microambiente que los TLRs. Hoy se sabe que la necrosis celular libera al medio extracelular moléculas que activan los TLRs. Así los TLRs también son sensores del daño celular porque responden a patrones moleculares asociados al daño (DAMPs). En la última década se ha producido un gran avance al elucidar las distintas vías de transducción de las señales intracelulares que se ponen en marcha tras la activación de los distintos TLRs, vías que son muy similares a las que se disparan tras la activación de la familia de los receptores de la IL-1. Entre las vías intracelulares que se activan tras la estimulación de los TLRs está el eje iniciado por la quinasa TAK1 que activa al complejo clásico IKK, subsecuentemente IKKβ fosforilará a p105-NFκB1 permitiendo la liberación de Cot/tpl-2 del complejo inactivo p105-NFκB1--ABIN2--Cot/tpl-2. Cot/tpl-2 es la única MAPKKK que activa a MKK1/2 y consecuentemente a Erk1/2, en estas condiciones. En esta Tesis Doctoral demostramos que tras la activación de los TLRs, Cot/tpl-2 vía Erk1/2 modula la activación de la vía de la PI3K a distintos niveles, llegando a controlar la traducción de los mensajeros Cap-dependientes. También demostramos que Cot/tpl-2 regula la inflamación estéril producida por la necrosis celular hepática tras una sobredosis de Acetaminofén (paracetamol). Finalmente, también mostramos que el eje Cot/tpl-2--MKK1/2--Erk1/2 juega un papel esencial en la activación de macrófagos por Adiponectina hacia un fenotipo pro-inflamatorio, una hormona secretada principalmente por el tejido adiposo con la capacidad de sensibilizar la acción de la insulina.

TLR family is pathogenic infection sensors by their capacity to recognize and be activated in response to pathogens associated molecular patterns (PAMPs). In recent years it has been shown that TLRs as well as other receptors included in the superfamily of receptors that recognize molecular patterns (PRRs), also recognize endogenous molecules that under physiological conditions are not physically in the same microenvironment that TLRs. Today it is known that cell necrosis releases into the extracellular media molecules that activate TLRs. So, TLRs are also cellular damage sensors because they respond to damage-associated molecular patterns (DAMPs). In the last decade there has been a breakthrough in elucidating the different intracellular signal transduction pathways upon activation of diverse TLRs, which are very similar to those generated after activation of the IL-1R family. Among the intracellular pathways that are activated after stimulation of TLRs is the axis of the TAK1 kinase that activates the classical IKK complex, IKK subsequently phosphorylates p105-NFκB1 triggering the release of Cot/tpl-2 from the p105-NFκB1--ABIN2--Cot/tpl-2 inactive complex. Cot/tpl-2 is the only MAPKKK that activates MKK1/2 and consequently Erk1/2 under these cell stimulation conditions. In this Thesis we show that upon TLRs activation, Cot/tpl-2 via Erk1/2 activation modulates PI3K pathway at different levels, modulating the translation of Cap-dependent messengers. We also show that Cot/tpl-2 regulates sterile inflammation produced by liver cell necrosis following an overdose of acetaminophen. Finally, we also show that the axis Cot/tpl-2--MKK1/2--Erk1/2 plays an essential role in the macrophages activation to a pro-inflammatory type following Adiponectin activation, a hormone secreted by adipose tissue primarily with the ability to sensitize the insulin action.

Índice

Abreviaturas y acrónimos

4E-BP1: proteína de unión a 4E 1

5´ TOP mRNA: mRNA con región de pirimidinas en el extremo 5´

5´ UTR: región 5´ no traducida

A20: enzima desubiquitinadora

ABIN: *A20-binding inhibitor of nuclear factor-kappa B*

AdipoR1: receptor 1 de la adiponectina

AdipoR2: receptor 2 de la adiponectina

AIM2: receptor 2 ausente en melanoma

AMPK: quinasa activada por AMP

AP-1: proteína activadora 1

APAP: Acetaminofén

APC: célula presentadora de antígeno

APN: adiponecinta

APPL1: *DCC-interacting protein 13-alpha*

Arg-1: arginasa 1

BMDM: macrófagos derivados de la médula ósea

Cap: m7GTP unido al primer nucleótido del mRNA en el extremo 5´

CCL: ligando quimioquina con motivos C-C

CD: conjunto de diferenciación

CLR: receptor tipo lectina C

COX2: ciclo-oxigenasa 2

CpG-DNA: ADN rico en islas CpG (citosina y guanina unidas por fosfatos)

CREB: elemento de respuesta por unión de cAMP

DAMPs: patrones moleculares asociados a daño

dsRNA: ARN de doble cadena

EC50: máxima concentración media efectiva

eEF: factor de elongación eucariota

eEF2K: quinasa de eEF2

eIF: factor de iniciación eucariota

Erk: quinasa regulada por señales extracelulares

FADD: *Fas-Associated protein with Death Domain*

FOXO1: factor de transcripción con dominio de unión a DNA tipo forkhead O1.

gAd: dominio globular de Adiponectina

GM-CFU: unidad formadora de colonias de granulocitos - macrófagos

GM-CSF: factor estimulador de colonias de granulocitos - macrófagos

GPCR: receptor acoplado a proteína G

Gr1: antígeno de diferenciación mieloide

HSP: proteínas de choque térmico

i.p.: intra-peritoneal

ICAM-1: molécula de adhesión intercelular-1

IFN: interferón

IgE: inmunoglobulina E

IGF-1: factor de crecimiento similar a insulina

IKK: quinasa que fosforila a IκB

IL: interleuquina o citoquina

IL-1R: receptor de IL-1

IP-10 (CXCL-10): quimioquina inducida por IFNγ 10

IRAK: quinasa asociada al receptor de la interleuquina 1

IRES: sitio interno de entrada del ribosoma

IRF: factor regulador de interferón

IRS-1: sustrato 1 del receptor de insulina

IκB: proteína reguladora inhibidora de NFκB

Jak: *Janus* quinasa

JNK: quinasa que fosforila a la proteína c-jun en el dominio N-terminal

KC (CXCL-1): quimioquina derivada de queranocitos

KD: sin actividad quinasa

KO: *knock out*

LDH: lactato deshidrogenasa

LPS: lipopolisacárido

LXR: receptor X de hígado

M1: macrófagos activados por la vía clásica

M2: macrófagos activados por la vía alternativa

m^7GTP: 7 metil GTP (guanosina tri-fosfato)

MAP2K: proteína quinasa quinasa activada por mitógenos

MAP3K: proteína quinasa quinasa quinasa activada por mitógenos

MAPK: proteína quinasa activada por mitógenos

MCP-1 (CCL2): ligando quimioquina 2 con motivos C-C

M-CSF: factor estimulador de colonias de macrófagos

MEF: fibroblastos embrionarios de ratón

MHC-I: complejo mayor de histocompatibilidad I

MHC-II: complejo mayor de histocompatibilidad II

MIF: media de intensidad de fluorescencia

MKK: MAP quinasa quinasa

mTORC: complejo diana de rapamicina de mamíferos

MyD88: gen de respuesta de la diferenciación mieloide primaria 88

NFAT: factor nuclear de activación de célula T

NF-κB: factor nuclear-κB

NK: linfocitos citolíticos naturales

NKT: células asesinas naturales T

NLR: receptor tipo NOD

NLRP3: receptor 3 con dominio NOD-, LRR- and pyrina

NO: óxido nítrico

NOS2: enzima óxido nítrico sintasa 2

PAMPs: patrones moleculares asociados a patógenos

PDK1: proteína quinasa dependiente de fosfatidil inositol

PGE_2: prostaglandina E_2

PI3K: quinasa que fosforila al fosfatidil inositol-4,5-bifosfato en la posición 3

PIP2: fosfatidil inositol-4,5-bifosfato

PIP3: fosfatidil inositol-3,4,5-trifosfato

PPAR: receptor activado por el proliferador de peroxisomas

PRR: receptores de reconocimiento de patrones

RAGE: receptor para productos finales de glicosilación

RE: retículo endoplasmático

RIP-1: proteína 1 de interacción con receptor

RLR: receptor tipo RIG-I

ROS: especies reactivas de oxígeno

RSK: quinasa ribosomal de S6

S6: proteína de la subunidad pequeña del ribosoma

S6K1: proteína quinasa de S6

SGK1: quinasa activada por suero/glucocorticoides

SIGIRR: single Ig IL-1-related receptor

SOCS: supresor de la señalización mediada por citoquinas

ssRNA: ARN de cadena simple

ST2: receptor de IL-33

STAT: transductor de señales y activador de la transcripción

TAB 1: proteína 1 de unión a TAK1

TAB 2: proteína 2 de unión a TAK1

TAK1: quinasa activada por TGFβ

TBK1: quinasa de unión a TANK 1

TCR: receptor de células T

TGFβ: factor de crecimiento tumoral 1

Th: linfocito T colaborador

TIR: Dominio del receptor Toll/IL-1

TIRAP: proteína adaptadora con dominio de unión al receptor TLR/IL-1

TLR: receptor tipo Toll

TNFα: factor necrótico de tumores α

TOLLIP: proteína de unión a TLR

TRAF6: factor asociado al receptor TNF 6

TRAM: molécula adaptadora asociada con TRIF

TRIAD3A: E3-ubiquitina ligasa

TRIF: adaptador con dominio TIR que induce la producción de IFNβ

tRNA: ARN-transferente

VCAM-1: molécula de adhesión vascular-1

Wt: salvaje

Introducción

1. El sistema inmune

Todo organismo vivo está expuesto continuamente a microorganismos infecciosos presentes en el entorno y necesita hacer frente a la invasión de éstos. Se define como sistema inmune al sistema responsable de hacer frente a agentes extraños. En vertebrados, la respuesta inmune está formada por dos componentes: la respuesta inmune innata y la respuesta inmune adaptativa. Ambos se activan frente a los microorganismos invasores generando respuestas específicas para eliminarlos. Sin embargo, en algunas ocasiones, problemas con el sistema inmune pueden producir enfermedades e infecciones [122, 192].

1.1. El sistema inmune innato

La inmunidad innata es la primera línea de defensa del organismo frente a la infección de agentes patógenos siendo el mecanismo de defensa más antiguo y conservado evolutivamente. Está presente en todos los organismos multicelulares y comprende los mecanismos de defensa bioquímicos y celulares presentes en el huésped. Las células del sistema inmune innato reaccionan frente a estructuras comunes conservadas en grupos de microorganismos distintos, gracias a receptores invariables codificados en la línea germinal. También pueden responder a moléculas de su propio organismo que fisiológicamente no se encuentran en el mismo entorno [122, 192].

Tradicionalmente se ha considerado que la respuesta inmune innata carece de memoria, respondiendo de la misma forma frente a infecciones sucesivas de un mismo patógeno. Sin embargo, recientemente se ha demostrado que algunos componentes de la respuesta inmune innata, como las NK, presentan capacidad de memoria [151].

Los principales componentes del sistema inmune innato son [146, 192] :

- Barreras mecánicas, físicas y químicas: comprenden epitelios y sustancias antimicrobianas, como enzimas o péptidos, sintetizadas en las superficies epiteliales que impiden la entrada de los patógenos en el organismo.

- Macrófagos, neutrófilos y células dendríticas: son células fagocíticas, principalmente los macrófagos, que engullen y digieren patógenos y partículas nocivas para el organismo. Además, las células dendríticas, principalmente, y los macrófagos son células presentadoras de antígeno (APCs) que activan a las células del sistema inmune adaptativo y todas ellas liberan citoquinas y quimioquinas.

- Mastocitos, basófilos y eosinófilos: son reguladores de la respuesta inflamatoria secretando mediadores químicos relacionados con la eliminación de parásitos y bacterias. Están implicadas en procesos alérgicos.

- Linfocitos citolíticos naturales (NK, Natural Killer): son leucocitos que atacan y destruyen células tumorales, y células infectadas por virus.

- Sistema del complemento: es el principal componente de la respuesta humoral de la respuesta inmune innata. Son más de 20 moléculas plasmáticas que opsonizan o recubren la superficie de los patógenos, dando como resultado la citólisis de las células infectadas y participando en el reclutamiento de células inflamatorias en el foco de la infección causando la muerte del patógeno.

1.2. El sistema inmune adaptativo

El sistema inmune adaptativo evolucionó en los vertebrados y permite una respuesta inmunitaria mayor y más específica. Posee capacidad de memoria, aumentando su respuesta en magnitud y capacidad de defensa con cada exposición sucesiva a un microorganismo determinado. Además, distingue moléculas específicas incluso de microorganismos muy relacionados entre sí, gracias a receptores específicos de antígeno que se generan al azar por reordenamiento e hipermutación de genes. Cada célula posee un determinado receptor y aquella célula que reconoce al antígeno se expande clonalmente en respuesta al estímulo [112, 122, 192].

Las principales células del sistema inmune adaptativo son [20, 40, 112, 192, 198] :

- Linfocitos T citotóxicos (Tc) ($CD3^+CD8^+$): reconocen antígenos acoplados a moléculas del complejo mayor de histocompatibilidad (MHC) de clase I.

- Linfocitos T colaboradores (Th) ($CD3^+CD4^+$): tras la estimulación por antígenos específicos, reconocidos por el MHC de clase II, producen citoquinas que estimulan la proliferación y activación de diversas células del sistema inmune como los linfocitos T citotóxicos y los macrófagos. Dependiendo del estímulo, se diferencian a tipo Th1, Th2 o Th17 que secretan grupos de citoquinas diferentes, generándose una respuesta inmune adaptativa determinada. Además, envían señales estimulantes adicionales requeridas generalmente para activar los linfocitos B, productores de anticuerpos. Los linfocitos T reguladores ($CD4^+CD25^+$) inhiben la respuesta inmune, evitando una respuesta inmune excesiva y manteniendo así la homeostasis del organismo.

- Células asesinas naturales T (NKT) ($NK1.1^{+}CD3^{+}$): comparten características comunes con células NK y linfocitos T.

- Linfocitos B ($CD19^{+}$): son las únicas células capaces de producir anticuerpos al reconocer antígenos extracelulares.

Aunque parece que el sistema inmune adaptativo existe solamente en vertebrados, se ha descubierto una molécula diferente, derivada de linfocitos, en vertebrados primitivos sin mandíbula, que se liga a antígenos de los patógenos de un modo similar a como lo hacen los anticuerpos y con el mismo grado de especificidad [2].

Los sistemas inmune innato y adaptativo forman un sistema de defensa integrado. Por un lado, la estimulación y el tipo de respuesta inmune adaptativa depende de la activación de la respuesta inmune innata; mientras que por otro lado, el sistema inmune adaptativo estimula al sistema inmune innato aumentando su capacidad antimicrobiana y a su vez, utiliza componentes del sistema inmune innato para eliminar los microorganismos [122, 192].

2. Los macrófagos en el sistema inmune

Los macrófagos son células heterogéneas y polivalentes, elementos clave en la defensa del organismo y en la inflamación, siendo uno de los componentes más importantes de la inmunidad innata. Entre los procesos en los que participan destacan: fagocitosis (función principal); inflamación, que comienza a través de receptores de membrana contra moléculas foráneas; presentación de antígeno, activando a linfocitos T colaboradores; y hemostasia, promoviendo la coagulación de la sangre.

La diferenciación de los macrófagos comienza en la médula ósea, donde una célula madre precursora se diferencia a una unidad formadora de colonias de granulocitos-macrófagos (GM-CFU), que es el precursor común de células mieloides: macrófagos, granulocitos (basófilos, eosinófilos y neutrófilos) y células dendríticas mieloides. Después, este precursor común se diferencia a monoblasto, posteriormente a pro-monocito y finalmente a monocito. Esta diferenciación está dirigida por los factores estimuladores de colonias, GM-CSF y M-CSF. A continuación, los monocitos se exportan al torrente sanguíneo y llegan a los tejidos diana atraídos por las quimioquinas producidas por el microambiente local. Una vez en el tejido, el monocito sufre un proceso de diferenciación a macrófago o a célula dendrítica. Los monocitos y los macrófagos expresan marcadores de superficie propios del linaje celular (CD115) y comunes con otras células inmunes (Ly6C, CD11a, CD11b y CD11c), cuyo grado de expresión varía según el estado de diferenciación/activación de estas células. Todos estos

precursores y células diferenciadas forman parte del sistema fagocítico mononuclear [59, 112, 135, 181].

En respuesta a una infección o a inflamación estéril inducida por daño tisular, los monocitos circulantes son reclutados hacia el foco de la inflamación y se diferencian a macrófagos [30, 59, 135]. Éstos poseen la plasticidad inherente que implica llevar a cabo distintos programas de activación específicos, según los distintos patrones de moléculas que se encuentran en los patógenos o en las células del huésped necrosadas y en función de las citoquinas presentes en el microambiente. Existen tres estados generales de activación de los macrófagos: activación clásica o M1, activación alternativa o M2 y desactivación [112, 129, 135] (Imagen 1).

2.1. Activación clásica de los macrófagos o M1

Tras el reconocimiento de patrones moleculares asociados a patógenos como el lipopolisacárido (LPS), componente mayoritario de la pared de las bacterias Gram negativas, y/o en presencia de citoquinas pro-inflamatorias producidas por las células Th1 y NK, principalmente interferón γ (IFNγ), los macrófagos se activan por la vía denominada clásica o M1 convirtiéndose en células efectoras que inhiben el crecimiento bacteriano y la replicación viral [112, 135, 198]. Los macrófagos M1 producen citoquinas pro-inflamatorias como los IFNα e IFNβ, TNFα, IL-12, IL-6 e IL-1β, quimioquinas proinflamatorias como KC (CXCL-1), IP-10 (CXCL-10) y CCL-5; además de inducir la expresión de la enzima óxido nítrico sintasa 2 o inducible (NOS2) que produce la molécula citotóxica óxido nítrico (NO). Estos mediadores inflamatorios inducen la fagocitosis y la destrucción de los microorganismos intracelulares fagocitados [110, 112, 198]. La activación de los macrófagos por la vía clásica, induce a su vez la polarización de los linfocitos colaboradores a tipo Th1, mediante la presentación de antígenos a los linfocitos Th a través del MHC-II y de las moléculas co-estimuladoras CD80 y CD86, así como por la producción de citoquinas pro-inflamatorias como IL-12 que estimulará a los linfocitos Th1 produciendo más IFNγ y generándose un sistema de retroalimentación positivo que aumenta la actividad citotóxica y la expresión de: MHC-II, CD80 y CD86 en la superficie de los macrófagos [198].

2.2. Activación alternativa de los macrófagos o M2

En presencia de algunas citoquinas anti-inflamatorias, como IL-4 e IL-13, producidas por linfocitos Th2, mastocitos y eosinófilos, los macrófagos son activados de forma alternativa o M2 para combatir parásitos. Esta vía alternativa de activación produce la inhibición parcial de la liberación de mediadores pro-inflamatorios y de la producción de óxido nítrico [55, 135]. El

gen inducido por los macrófagos M2 más conocido es Arg-1, que compite con la NOS2 por el mismo sustrato, la L-arginina. Mientras que NOS2 forma óxido nítrico, el enzima Arg-1 sintetiza ornitina y poliaminas, induciendo el crecimiento celular y la reparación tisular mediante la producción de colágeno [55, 135]. La activación alternativa de los macrófagos no antagoniza simplemente las vías de señalización inducidas por la respuesta M1, ya que por ejemplo la citoquina IL-4 también estimula la endocitosis y la expresión de MHC-II, estimulando la presentación de antígeno a los linfocitos T colaboradores [55]. Además, los macrófagos tipo M2 inducen la proliferación de las células Th2, la fagocitosis de desechos celulares y están involucrados en el desarrollo de alergia y asma, estimulando a eosinófilos y basófilos, y activando la producción de IgE por los linfocitos B [45, 55, 129, 135].

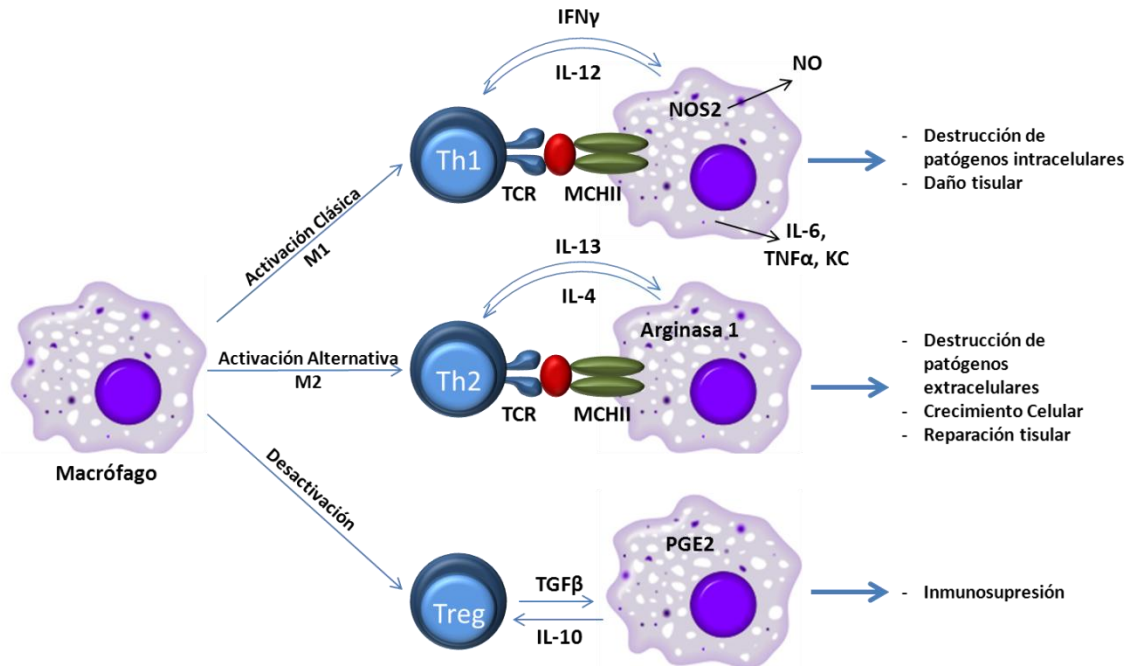


Imagen 1: Representación de los distintos tipos de activación de los macrófagos.

2.3. Desactivación de los macrófagos

La desactivación de los macrófagos es un proceso activo y controlado que suprime los programas inmunogénicos e inflamatorios de los macrófagos resultando en la resolución de la inflamación, evitando así el daño celular [112, 129]. Inicialmente, las moléculas pro-inflamatorias secretadas por los macrófagos sirven para eliminar los agentes extraños, pero una respuesta excesiva podría culminar en daño tisular y sepsis, por lo que se necesita un sistema capaz de controlar la magnitud y duración de las respuestas inflamatorias [112, 129]. La desactivación de los macrófagos está inducida principalmente por las citoquinas anti-inflamatorias IL-10 y TGFβ, producidas por los linfocitos T reguladores, y por glucocorticoides.

Estos componentes inhiben potentemente la transcripción génica de NOS2, ciclooxigenasa 2 (COX2) y TNFα, y la presentación antigénica a los linfocitos T colaboradores, mediante la inhibición de la expresión de MHC-II. Además, la activación de los macrófagos por IFNγ, IL-4 y/ó LPS, induce rápidamente la expresión de proteínas supresoras de la señalización de las citoquinas (SOCS), que inhiben las vías de señalización inducidas por citoquinas como IL-6, IL-10, IL-12, IFNα e INFγ [215] y por LPS. A su vez, la producción de la proteína pro-inflamatoria COX2 en los macrófagos activados induce la formación de prostaglandina E_2 (PGE_2) que estimula la producción de IL-10. De esta forma los macrófagos controlan la homeostasis celular mediante sistemas de retroalimentación negativa [58, 112, 129].

Los macrófagos no están permanentemente en uno de los tres estados descritos anteriormente, sino que son células plásticas capaces de polarizar a un tipo específico dependiendo del microambiente local [45, 135].

3. La inflamación

Es la respuesta del sistema inmune de un organismo al daño causado a sus células y tejidos. La inflamación puede ser desencadenada por un agente tanto externo como interno al organismo. En función del tiempo de duración, y del tipo celular principal que participa en la resolución del evento inflamatorio, podemos clasificar la respuesta inflamatoria en aguda o crónica. La vía inflamatoria consta de: inductores, sensores, mediadores y efectores. Los agentes inductores son las señales que inician la respuesta inflamatoria activando sensores especializados que terminan provocando la producción de mediadores de distinta naturaleza. Estos, son capaces de alterar los estados funcionales del órgano o el tejido afectado (que son los efectores de la inflamación), para que puedan adaptarse y resolver así, el estado inflamatorio [121].

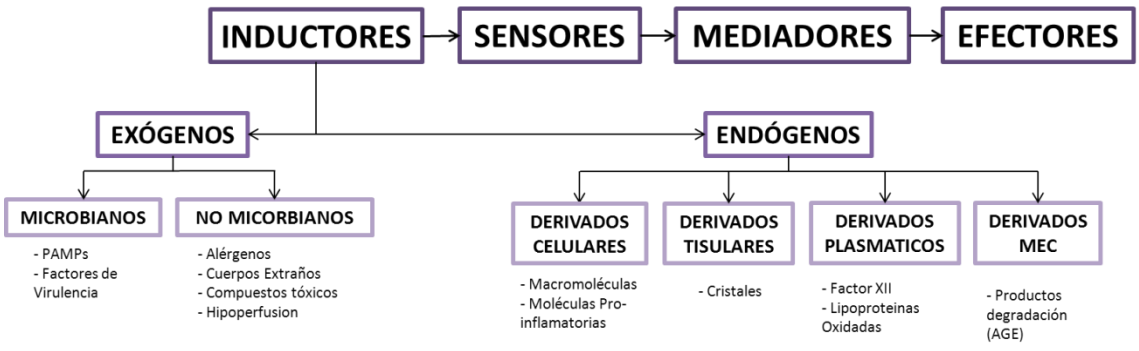


Imagen 2: Tipos de inductores de la respuesta inflamatoria. MEC: matriz extracelular. Modificado de [121]

3.1. Inductores de la inflamación

Se clasifican en dos grandes grupos dependiendo de su naturaleza: Endógenos y Exógenos (Imagen 2):

3.1.1. Inductores Exógenos

Existen dos tipos: los de naturaleza microbiana y los no microbianos:

▪ Inductores Exógenos Microbianos: patrones moleculares asociados a patógenos (PAMPs) y los factores de virulencia.

Los **PAMPs** son un conjunto definido y limitado de patrones moleculares muy conservados que se encuentran presentes en un gran número de microorganismos, que pueden ser reconocidos por la superfamilia de receptores denominados receptores de reconocimiento de patógenos (PRRs) (ver sección 3.2) [121].

Los **factores de virulencia**, éstos a diferencia de los PAMPs no son reconocidos por receptores específicos. Los efectos adversos derivados de la actividad de los factores de virulencia, como la diseminación celular o la inactivación de antibióticos son los responsables de desencadenar la respuesta inflamatoria [121].

▪ Inductores Exógenos No Microbianos: incluye alérgenos, irritantes, cuerpos foráneos y compuestos tóxicos.

Hay alérgenos que imitan la actividad virulenta de ciertos parásitos, mientras que otros pueden actuar como irritantes en el epitelio de la mucosa. Los cuerpos extraños son partículas indigeribles que, o bien son demasiado grandes para ser fagocitadas, o causan daño en la membrana de los macrófagos. Partículas de sílice y de asbesto son dos ejemplos de cuerpos extraños [121].

3.1.2. Inductores Endógenos

Son señales provocadas por estrés, o bien por daño o mal funcionamiento de un órgano o tejido. Estas moléculas están agrupadas dentro de los patrones moleculares asociados al daño (DAMPs) y su número aumenta continuamente [153].

La célula tiene una extraordinaria capacidad de adaptación. Cuando un agente externo o interno altera su fisionomía, sobrepasando los límites de dicha adaptabilidad, surge la lesión celular, pudiendo ser reversible o irreversible [77]. Ante diversos estímulos, las células experimentan unos cambios que les sirven para adecuarse a la situación como: atrofia;

hipertrofia; hiperplasia; o metaplasia. Cuando todos los mecanismos de adaptación y de resistencia se han agotado sobreviene la muerte celular.

Existen dos mecanismos de muerte diferentes: **1**. La apoptosis (muerte celular programada), controlada genéticamente y que se lleva a cabo de forma ordenada sin liberación de moléculas citoplasmáticas [77, 117]. **2**. Necrosis, proceso irreversible en el que no se mantiene la integridad de la membrana plasmática, dando lugar a la liberación de elementos citoplasmáticos, desnaturalización de las proteínas por acción de los lisosomas (autólisis) o proveniente de enzimas líticas de leucocitos vecinos (heterolisis), produciéndose la generación de los DAMPs [77, 117].

El hígado es uno de los órganos más importantes por su actividad metabólica. Es un órgano glandular al que se le adjudican funciones como: la síntesis de proteínas plasmáticas, función desintoxicante o almacenaje de vitaminas y glucógeno. También es el responsable de eliminar de la sangre compuestos que puedan resultar nocivos para el organismo. El alcoholismo, la ingestión de toxinas y la sobredosis de fármacos son las causas más comunes, que pueden originar una lesión hepática. Según el Consejo Nacional de Alcoholismo y Farmacodependencia, el uso nocivo del alcohol es causa de la muerte de 2,5 millones de personas al año, causa enfermedades y lesiones a muchos millones más, y afecta cada vez más a nuevas generaciones de bebedores en los países en desarrollo. Por otra parte, fármacos como el Acetaminofén (APAP, paracetamol), uno de los analgésicos y antipiréticos más vendidos en América del Norte y Europa, causa aproximadamente el 50% de las lesiones hepáticas agudas [18]. Estos agentes externos causan una alteración de la función mitocondrial promoviendo un aumento de las especies reactivas de oxígeno (ROS), que a su vez conducen a una pérdida total de la función mitocondrial. La falta de equilibrio en los pares redox, como el glutatión (GSH-GSSG); la pérdida del potencial de membrana

Tabla 1: Diferentes estímulos estériles y patologías asociadas. Modificada de [32]

Señal Inflamatoria Estéril	Patología Asociada
HSPs	Necrosis y lesión celular
Proteínas S100	Necrosis y lesión celular
RNA	Necrosis y lesión celular
DNA	Necrosis y lesión celular
Ácido hialurónico	Necrosis y lesión celular
Biglicanos	Necrosis y lesión celular
DNA (mitocondrial)	Necrosis y lesión celular
β-Amiloide	Enfermedad de Alzheimer
IL-1α	Necrosis y lesión celular
IL-33	Necrosis y lesión celular

mitocondrial, la producción de moléculas de alta energía (ATP o succinil-CoA); o la toxicidad inducida por iones quelantes, como el Fe^{+2} o Fe^{+3}, desembocan en una alteración de los niveles de ROS con pérdida de la integridad mitocondrial, y finalmente la perdida de la integridad celular, liberando al medio todos sus componentes [79]. La inflamación como consecuencia de un trauma, lesión inducida químicamente o por isquemia-reperfusión, ocurre en ausencia de ningún microorganismo y es conocida como inflamación estéril [32].

Los causantes de esta inflamación estéril pueden dividirse en dos grupo: intracelulares, como proteínas de choque térmico (HSPs) [12, 33, 156, 194]; metabolitos de Purina, como el ATP [22] o ácido úrico[91]; y extracelulares, que derivan de la proteólisis de enzimas de remodelación y/o reparación de tejido como el ácido hialurónico [78, 171] o los biglicanos [8, 170]. Asimismo, formas activas de citoquinas y quimioquinas pro-inflamatorias, como IL-1α [31, 41, 90] o IL-33 [24, 133], pueden liberarse al exterior y desencadenar una respuesta inflamatoria estéril, aunque no se consideren propiamente DAMPs [32] (Tabla 1).

El reclutamiento al foco inflamatorio de leucocitos, la producción de citoquinas y quimioquinas, y la inducción de una respuesta inmune adaptativa sugiere que tanto DAMPs como PAMPs podrían utilizar receptores y vías de señalización comunes [32].

3.2. Sensores de la inflamación

Los PRRs se encuentran altamente conservados entre diferentes organismos y no solo reconocen PAMPs, sino que también son capaces de reconocer DAMPs [32]. Los distintos tipos de receptores PRRs los podemos clasificar como:

• Receptores tipo lectina C (CLRs): superfamilia de proteínas transmembrana, con uno o más dominios c-lectina de unión a carbohidratos.

• Receptores tipo NOD (NLRs): proteínas localizadas en el citoplasma, caracterizadas por la presencia de un dominio NOD (nucleotide oligomerization domain) conservado y repeticiones ricas en leucina (LRRs).

• Receptores tipo RIG-I (RLRs): proteínas solubles localizadas en el citoplasma implicadas principalmente, junto con los TLRs, en la respuesta antiviral.

• Receptores tipo Ausencia en Melanoma 2 (AIM2): caracterizados por tener dominios de pirinas y dominios HIN de unión a DNA involucrados en el reconocimiento de DNA microbiano intracelular [193].

• Receptores tipo Toll (TLRs): son proteínas unidas a membrana, capaces de reconocer una gran variedad de ligandos microbianos. De este tipo de receptores hablaremos en profundidad más adelante en la sección 4.

3.3. Mediadores de la inflamación

En respuesta a los inductores inflamatorios, los distintos tipos celulares implicados en la inflamación, como plaquetas, células dendríticas, mastocitos, neutrófilos y/o macrófagos, van a secretar una serie de moléculas de distinta naturaleza química (mediadores químicos de la inflamación), que van a modular el proceso por su capacidad de [121]: modular la vasculatura del área inflamada; alterar la funcionalidad de tejidos y órganos; modular el reclutamiento de células al foco inflamatorio; y activar a distintos tipos celulares para que a vez secreten nuevos mediadores inflamatorios.

La gran mayoría de estos mediadores se sintetizan y almacenan hasta el momento de su liberación, algunos circulan como precursores inactivos por el plasma, y otros se sintetizan inmediatamente tras el estímulo adecuado. Los podemos diferenciar en 7 grupos según sus propiedades bioquímicas [121]:

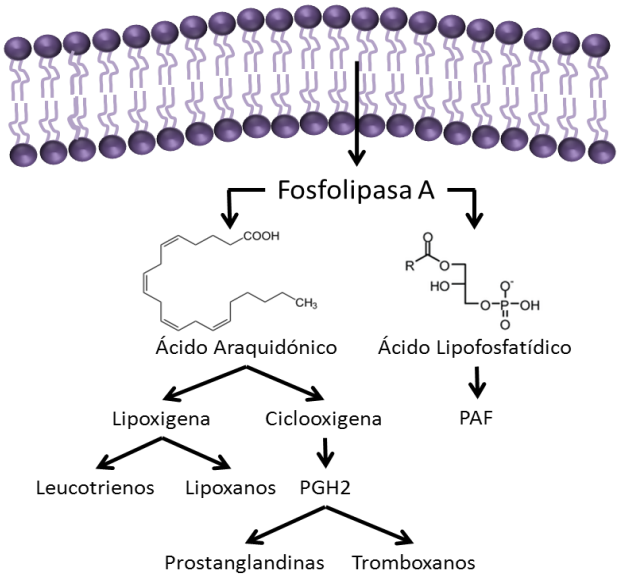


Imagen 3: Metabolismo de los mediadores lipídicos por la fosfolipasa A.

1. Aminas Vasoactivas (Histamina y Serotonina): Causan un cambio en la permeabilidad vascular; vasodilatación o vasoconstricción, dependiendo del contexto.

2. Péptidos Vasoactivos (Substancia P, fibrinopéptido A): Se encuentran como formas activas en vesículas secretoras o se activan por proteólisis en el fluido extracelular a partir de precursores inactivos, causando vasodilatación y aumento de la permeabilidad vascular.

3. Fragmentos del Complemento (C3a, C4a y C5a): Se pueden producir tras la activación de varias vías del complemento, reclutan granulocitos y monocitos al foco inflamatorio e inducen la desgranulación de mastocitos, afectando así, a la vasculatura.

4. Mediadores lipídicos (Eicosinoides y factor activador de plaquetas): derivados de fosfolípidos de la membrana lipídica interna (Imagen 3). La fosfolipasa A citosólica, tras activarse por un aumento local de iones Ca^{+2}, genera Ácido araquidónico y Ácido lisofosfatídico, precursores de mediadores lipídicos. El primero puede metabolizarse por las ciclooxigenasas (COX1 y COX2) para dar prostanglandinas (PGE_2 y PGI_2) y tromboxanos; o por las lipoxigenasas, generando leucotrienos y lipoxinas. Tienen acción vasodilatora e hiperalgésica (PGE_2). Por otra parte, los factores de activación de plaquetas (PAFs) se generan por acetilación del Ácido lisofosfatídico y pueden modular varios procesos durante la respuesta inflamatoria como la vasodilatación o vasoconstricción, el reclutamiento de leucocitos al foco, el aumento de la permeabilidad vascular y la activación de plaquetas.

5. Citoquinas: producidas por una gran variedad de tipos celulares, sobre todo por macrófagos y mastocitos. Tienen diferentes papeles en la respuesta inflamatoria incluyendo la activación del endotelio y la inducción de la fase aguda.

6. Quimioquinas: producidas por un gran número de células en respuesta a los inductores, controlan la extravasación de leucocitos y la quimiotaxis entre los tejidos afectados.

7. Enzimas proteolíticos: ayudan a la defensa del huésped, a la remodelación tisular y a la migración de leucocitos.

3.4. Efectores de la inflamación

Los efectores inflamatorios son las células y los tejidos afectados por el evento inflamatorio. En función de los inductores que desencadenen la respuesta, y los mediadores que modulen el proceso inflamatorio, los efectores actuarán con el fin de resolver el episodio inflamatorio y volver a recuperar la homeostasis inicial en el tejido afectado.

4. Los receptores tipo Toll (TLRs)

Como se ha descrito anteriormente, tanto PAMPs como DAMPs son reconocidos por receptores de reconocimiento de patógenos (PRRs). El principal tipo de PRR son los receptores tipo Toll (TLRs) [75, 106, 148].

El receptor tipo Toll es un receptor transmembrana tipo I, conservado evolutivamente y presente en insectos, plantas y mamíferos. El dominio extracelular (LRR) con motivos ricos en leucina está implicado en el reconocimiento de los patógenos; mientras que la región citoplasmática contiene un dominio TIR (Toll/IL-1R) responsable de la señalización celular, también presente en los receptores de la IL-1 [130]. En mamíferos, se han identificado 12

miembros de la familia TLR, siendo los TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7 y TLR9 los más estudiados. Están localizados en distintos compartimentos celulares: membrana plasmática, RE y endosomas [85, 179]. Los TLR3, TLR7, TLR8 y TLR9 se encuentran ubicados en endosomas, y por su capacidad como sensores de ácidos nucleicos, son muy importantes en la detección de virus y DNA del huésped que no son capaces de ser reconocidos por otros sistemas [89]. Por otra parte, una respuesta inmune aberrante puede ser la responsable de la patogénesis autoinmune, inflamaciones crónicas y enfermedades infecciosas [49, 88].

Cuando un ligando se une a su receptor TLR, éste dimeriza induciéndose la conformación necesaria para reclutar moléculas implicadas en la transmisión de la señal. Según las moléculas adaptadoras que reclute, la señalización celular mediada por los TLRs se divide en dos grandes grupos: señalización MyD88-dependiente y señalización MyD88-independiente [84, 85, 179]:

4.1. Señalización MyD88-dependiente

Excepto el TLR3, todos los TLRs activos reclutan el adaptador MyD88, que a su vez recluta a, IRAK4 e IRAK1. IRAK4, al fosforilar a IRAK1, facilita el reclutamiento de TRAF6 al complejo. El complejo IRAK1-TRAF6 se disocia del receptor e interacciona con la MAP quinasa quinasa quinasa TAK1 produciendo su activación [107]. TAK1 activa las vías de las MAP quinasas p38α y JNK1/2 y al complejo IKK. El complejo IKK activo conduce a la activación de la vía de la MAPK Erk1/2 y a la fosforilación y degradación de IκB vía proteasoma, liberando al factor de transcripción NFκB. Las MAPKs y NFκB activan la transcripción de mediadores pro-inflamatorios como el TNFα, IL-1, IL-6 [84, 130, 179], y otras citoquinas, quimioquinas pro-inflamatorias y moléculas co-estimuladoras como NO, a través de los factores de transcripción AP-1 y CREB (Imagen 4) [20, 110, 179].

4.2. Señalización MyD88-independiente

Por otro parte, el TLR3 y el TLR4, en endosomas tardíos, reclutan al adaptador TRIF, que interacciona con TBK1. TBK1 activada interacciona con IKKε y fosforila al factor de transcripción IRF3. IRF3 fosforilado dimeriza y se transloca al núcleo induciendo la transcripción de genes IFN tipo I. Además, TRIF interacciona con TRAF6 activando a TAK1 y por tanto induce también la activación del factor de transcripción NFκB y de las MAP quinasas (Imagen 4) [210].

La producción de IFN tipo I en respuesta a la activación de los TLRs, induce a su vez la activación autocrina y paracrina de la vía de señalización Jak/STAT1. La activación de la

señalización Jak/STAT1 induce la dimerización del factor de transcripción STAT1 que se transloca al núcleo activando la transcripción de genes diana, como por ejemplo NOS2, IRF1, IFNγ, CCL-5 o IP-10 [140].

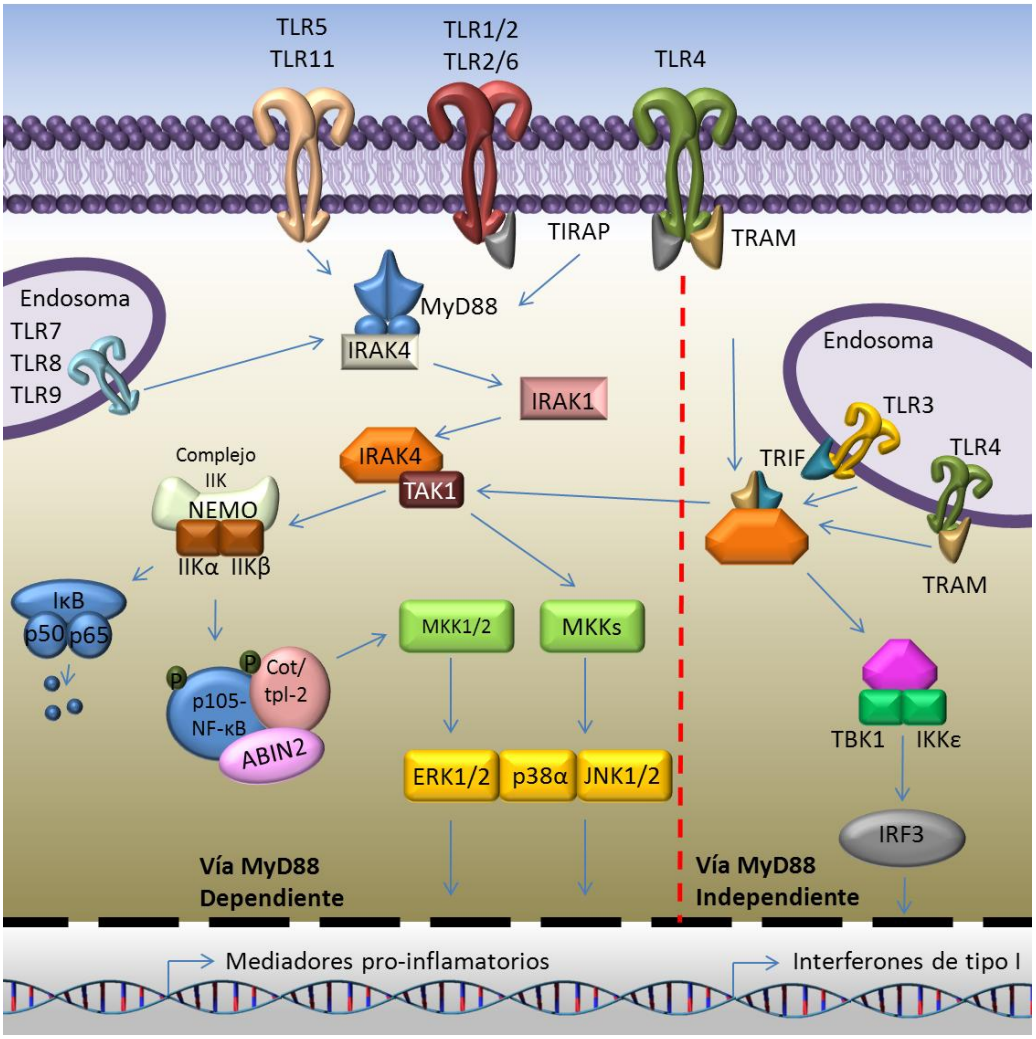


Imagen 4: Señalización mediada por los diferentes TLRs: MyD88-dependiente y MyD88-independiente. Modificada de [130].

4.3. Señalización de la fosfoinositol 3 quinasa (PI3K)

La PI3K es un enzima de la familia de quinasas lipídicas muy conservadas evolutivamente y que responde a una gran variedad de estímulos como nutrientes, hormonas y factores de crecimiento. Juega una función no redundante en múltiples procesos celulares desde crecimiento celular y proliferación hasta migración y producción de citoquinas. La unión de los ligandos a receptores activa la PI3K, que consecuentemente desencadena la generación de PIP3 por la unión de un grupo fosfato al carbono 3 del resto de Inositol del PIP2. El aumento local de PIP3 facilita la translocación de Akt a la membrana plasmática. Su activación comienza

con la fosforilación de su T308 por PDK1. Una completa activación de Akt requiere una segunda fosforilación en S473 mediada por mTORC2 [48, 79]. Akt activa puede fosforilar a FOXO1, factor de transcripción nuclear que media la apoptosis y la parada del ciclo celular [61]. Akt también es capaz de promover la activación del complejo diana de rapamicina en mamíferos (mTOR).

En condiciones basales, la actividad quinasa de mTORC1 se encuentra inhibida por la acción del heterodímero supresor de tumores TSC1-TSC2 conservado desde *Drosophila* a mamíferos. La activación de Akt, en la T308 es suficiente para la fosforilación de TSC2, lo que conduce a la inhibición de la función de TSC1-TSC2, y la consecuente activación de mTORC1. La vía de señalización de Erk1/2 también es capaz de activar mTORC1 indirectamente mediante la quinasa RSK (p90 RSK) [114]. Las principales dianas de mTORC1 son componentes de la maquinaria de traducción, incluidas la proteína inhibidora 4E-BP1 y la proteína S6 quinasa (p70 S6K o S6K1). La fosforilación de 4E-BP1 por mTORC1 da lugar a la disociación del complejo 4E-BP1--eIF4E, permitiendo la formación del complejo de iniciación de la traducción, esencial para la traducción de los mRNAs Cap-dependientes. La quinasa S6K1 fosforila a la proteína S6 de la subunidad pequeña del ribosoma 40S, que está relacionada con un ensamblaje más eficiente del ribosoma con el complejo de iniciación de la traducción. S6K1 también fosforila a la quinasa eEF2K inhibiéndola. eEF2K fosforila e inhibe al factor de elongación eEF2 que media el paso de translocación en la elongación de la cadena peptídica donde el ribosoma migra a lo largo del mRNA [155]. Estas dos dianas de S6K1 pueden ser fosforiladas también por RSK [3].

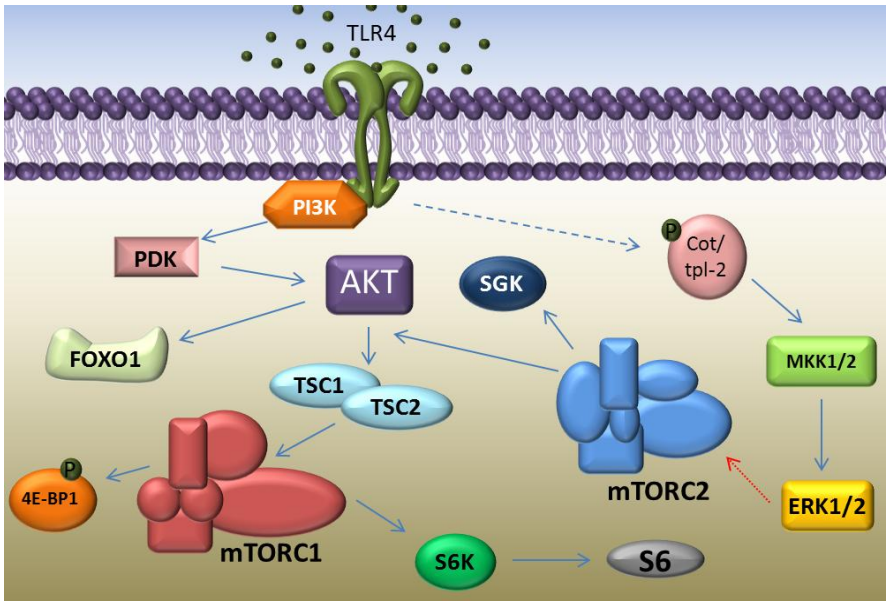


Imagen 5: Representación esquemática de la vía PI3K/Akt.

mTORC1, no solo activa la traducción de mRNAs Cap-dependientes, sino que también activa la traducción de una población específica de mRNAs con motivos oligopirimidina terminales en el 5' (5´ TOP mRNAs) en los que se genera una estructura secundaria que impide el acceso de la maquinaria de inicio de la traducción al AUG iniciador. El número de transcritos individuales que pertenecen a la familia de los 5´ TOP mRNA es pequeño, pero su abundancia en las células puede representar el 20% del total del mRNA celular [125].El subgrupo de mRNAs que son regulados específicamente por mTORC1 son casi exclusivamente aquellos con motivos 5'TOP [184].

5. Vías de señalización de las MAP quinasas. MAP3K8 Cot/tpl-2

Las proteínas quinasas activadas por mitógenos, también llamadas MAP quinasas (MAPKs), constituyen una familia de proteínas estimuladas por factores de crecimiento, mitógenos, estrés y citoquinas pro-inflamatorias principalmente. Estas proteínas juegan un papel fundamental en la transducción intracelular de señales llevando a cabo multitud de procesos celulares como desarrollo, crecimiento y diferenciación celular, apoptosis e inflamación, a través de otras proteínas quinasas, así como a fosfolipasas, factores de transcripción, componentes de la maquinaria apoptótica y proteínas del citoesqueleto [95].

Imagen 6: Representación esquemática de las diferentes cascadas de señalización de las MAPKs.

Cada subfamilia está compuesta por un módulo de señalización de tres quinasas evolutivamente conservadas que se activan de forma secuencial: las MAPKKK activan a las MAPKK y estas a las MAPK, mediante fosforilaciones en residuos de serina y treonina o de treonina y tirosina [95] (Imagen 6).

En el sistema inmune son muchas las MAP3Ks capaces de activar diferentes vías de MAPKs en respuesta a estímulos específicos, generando repuestas específicas. Este es el caso de la serina/treonina MAP3K8 Cot/tpl-2, objetivo central de esta Tesis doctoral.

5.1. Caracterización y descubrimiento de Cot/tpl-2

A principio de la década de 1990, 3 grupos diferentes descubrieron Cot/tpl-2 como un oncogén: **1**, al transformar, in vitro, una línea celular embrionaria de hámster con DNA genómico de una línea celular proveniente de carcinoma humano de tiroides [128]; **2**, el gen murino homólogo de Cot, tpl-2, fue identificado como una proteína truncada en el dominio C-terminal, a consecuencia de la inserción del provirus de la leucemia de Moloney (MoMuLV) en rata [149]; y **3**, el locus de *Tpl2*,se identificó como un lugar de inserción para el Virus de tumor de mama en ratones (MMTV) asociado con la inducción de carcinomas de mama en ratones [44]. El resultado fue una proteína truncada en el extremo C-terminal, lo que sugirió que este dominio jugaba un papel importante en la regulación de su actividad [54].

Cot/tpl-2 se localiza principalmente en el citoplasma. Debido a 2 inicios de traducción diferentes se generan 2 isoformas de 58 y 52 KDa [6]. Las versiones oncogénicas, murina y humana, no expresan el dominio C-terminal de la proteína implicado en la inhibición de su actividad quinasa específica. Este dominio contiene un degrón usado como señal para su degradación vía proteasoma. Así, *Cot/tpl-2* truncada presenta mayor vida media y mayor actividad específica, incrementándose su actividad quinasa [25, 53]. Aunque siempre se ha relacionado a Cot/tpl-2 con un oncogén, puede actuar como un supresor de tumores [37, 189].

Tras los datos comentados, se pensó que de la forma proto-oncongénica de Cot/tpl-2 podría ser un regulador de señales proliferativas, pero posteriormente se reportó que ratones deficientes en Cot/tpl-2 eran resistentes a un tipo de síndrome de shock séptico debido a la disminución de la producción de TNFα entre otras citoquinas [39]. Actualmente está aceptado que la función fisiológica más importante de Cot/tpl-2 es la regulación de la MAPK Erk1/2 en respuesta a la activación por la súper-familia de receptores TLR/IL-1 y TNF-R [54].

5.2. Papel fisiológico de Cot/tpl-2

5.2.1. Activación de Cot/tpl-2 por estímulos extracelulares

En condiciones basales, Cot/tpl-2 forma un complejo estable e inactivo con p105-NFκB y ABIN2 entre otras proteínas [14, 100, 199]. p105-NFκB interacciona con dos dominios de Cot/tpl-2: con el dominio C-terminal donde se localiza la señal de degradación, protegiendo a Cot/tpl-2 de la degradación vía proteasoma; y por otro lado, con el dominio quinasa, impidiendo que Cot/tpl-2 ejerza su función sobre su sustrato [13]. Además la interacción de Cot/tpl-2 con ABIN2 también es necesaria para proteger a Cot/tpl-2 de su degradación vía

proteasoma [100], sin bloquear su actividad quinasa [147, 200]. Además, diversos estudios proponen la interacción del complejo con otras proteínas [169].

La estimulación de la actividad quinasa de Cot/tpl-2 necesita, al menos, de dos pasos de regulación: (1) la liberación del complejo con p105-NFκB. Tras la estimulación de la superfamilia de TLR/IL-1R, se activa el complejo IKK que llevará a cabo la fosforilación de p105-NFκB, por IKKβ, dando lugar a su proteólisis parcial, vía proteasoma [66, 166, 195]; y (2) la auto- y/o trans-fosforilación en los residuos T290 [111] y S400 [158] (Imagen 7). Además, Cot/tpl-2 también se autofosforila en el residuo S62, aumentando su capacidad de activar a su sustrato MKK1/2 [176]. Una vez que Cot/tpl-2 se disocia del complejo y se activa, es degradada rápidamente vía proteasoma [205, 206]. Destacar que Cot/tpl-2 tiene como único sustrato a MKK1/2 activando consecuentemente Erk1/2.

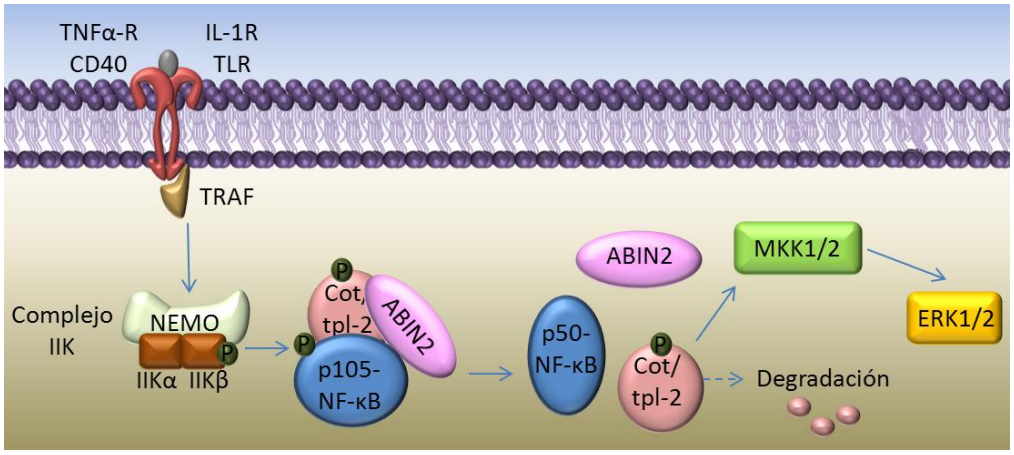


Imagen 7: Mecanismo de activación fisiológica de Cot/tpl-2.

5.2.2. Vías de señalización intracelulares inducidas por Cot/tpl-2

Mediante estudios de sobreexpresión de Cot/tpl-2 se vió que tanto la proteína salvaje (Wt) como la forma truncada, promueven la activación de diferentes MAPKs, como Erk1/2, JNK1/2, p38α y Erk5 y de diversos factores de transcripción como AP-1, NFAT y NFκB [9, 35, 150, 165, 166, 169, 187, 188, 195]. Estudios posteriores han llevado a proponer que la señalización mediada por Cot/tpl-2 es específica de estímulo y tipo celular. Por ejemplo, estudios en macrófagos *Tpl2*$^{-/-}$ revelan en respuesta a LPS que Cot/tpl-2 juega un papel importante en la activación de MEK1/2 y Erk1/2, pero no en la de p38, JNK o NFκB [13, 206]. La activación de Erk1/2 también esta mediada por Cot/tpl-2 en macrófagos a través de los receptores TLR2, TLR9 y TNFα-R; y de CD40 en células B [42, 83, 125]. También, es necesaria para la activación de Erk1/2 y JNK en MEF estimulados con TNFα o IL-1β; y de p38 en células dendríticas estimuladas con CpG o LPS [36, 83].

5.2.3. Implicación de Cot/tpl-2 en inmunidad e inflamación

Cot/tpl-2 es la única quinasa capaz de fosforilar Erk1/2 en respuesta a la activación de los receptores TLR2, TLR3, TLR4, TLR7, TLR9 y TNFα en macrófagos y células dendríticas, del receptor CD40 en linfocitos B, y del receptor de IL-1β en células HeLa [11, 23, 39, 42, 83, 108, 159]. La eliminación de Cot/tpl-2 disminuye la expresión de COX2 y la secreción de TNFα e IL-1β en macrófagos estimulados con LPS; la secreción de IgE en células B y la producción de IL-8 y MIP- 1β en células HeLa estimuladas con IL-1β [39, 42, 43, 159].Por otra parte, Cot/tpl-2 ha emergido como un regulador negativo de la respuesta inmune tipo Th1 al demostrarse que ratones deficientes en la Cot/tpl-2 muestran mayor producción de IL-12 y de IFNβ en respuesta a la activación del TLR4 por LPS y del TLR9 por CpG que los ratones Wt [83, 177].

Estudios recientes muestran que Cot/tpl-2 desempeña un papel importante en el desarrollo de procesos inflamatorios *in vivo*. Así, ratones Cot/tlp-2 KO presentan una reducción en la inflamación pancreática inducida por caerulina [196]. Además, Cot/tpl-2 está implicada en la inflamación periférica aguda y en la hipernocicepción inducida por zymosan [175], y juega un papel importante en el inicio de la enfermedad de Crohn [93].

5.3. Cot/tpl-2 como diana de drogas anti-inflamatorias

Las MAP3Ks Cot/tpl-2 y Raf son capaces de promover la fosforilación múltiples sustratos, a través de la activación de MKK1/2-Erk1/2. Sin embargo, mientras que Raf activa Erk1/2 en respuesta a la activación de señales proliferativas, Cot/tpl-2 activa Erk1/2 en respuesta a la activación de los TLRs y citoquinas pro-inflamatorias, por lo que el papel de Cot/tpl-2 en inmunidad e inflamación no puede ser remplazado por ninguna otra proteína. Esto convierte a Cot/tpl-2 en una buena diana para desarrollar nuevas drogas anti-inflamatorias [26, 51, 52]. Varios grupos y compañías están desarrollando inhibidores específicos de la actividad quinasa de Cot/tpl-2 así como ribozimas que degradan específicamente el mRNA de Cot/tpl-2 [27, 60, 82, 104, 105]. Futuros estudios determinarán si estos compuestos inhiben específicamente su actividad y pueden ser utilizados *in vivo.*

6. Adiponectina (APN)

La APN es una hormona proteica, producida predominantemente por los adipocitos [116]. Fue identificada entre 1995 y 1996 por cuatro grupos investigadores diferentes. Fue clonada a partir de líneas celulares murinas [72, 172]. Por otro lado, se reportó que era el

transcrito más abundante en adipocitos en biopsias de tejido adiposo humano [116]. Finalmente se aisló del plasma humano por cromatografía de afinidad [136].

6.1. Estructura y aspectos generales

La APN es una proteína de 28-30 KDa con 247 Aas y 4 dominios bien diferenciados [17] (Imagen 8, A): dominio amino terminal que contiene la señal secretora; región variable característica de especie constituida por 28 Aas; dominio tipo colágeno (cAd) constituido por 22 tripletes glicina-X-tirosina (G-X-Y); y dominio globular situado en la región carboxy-terminal.

Tras su síntesis, el monómero de APN oligomeriza por el dominio colágeno para formar homotrímeros (Imagen 8, B). A partir de estos, pueden formarse estructuras superiores de peso medio (MMW) y alto (HMW) de hasta 400 kDa [144]. Además, en plasma se han identificado pequeñas cantidades del fragmento globular aislado (gAd), que resulta de la ruptura proteolítica del homotrímero. Estudios con formas mutantes recombinantes de APN, que solo pueden generar la forma trimérica, demuestran que es la forma con mayor actividad biológica. Estos resultados sugieren que las formas MMW y HMW pueden actuar como precursores de la forma trimérica [144].

Análisis funcionales demuestran que la APN recombinante más activa es la sintetizada en células de mamífero, posiblemente debido a las modificaciones post-traduccionales que se dan en el dominio colágeno como la O-glucosidación con ácido disiálico [167]; la hidroxilación y la glucosilación, pudiéndose generar hasta ocho isoformas diferentes [204].

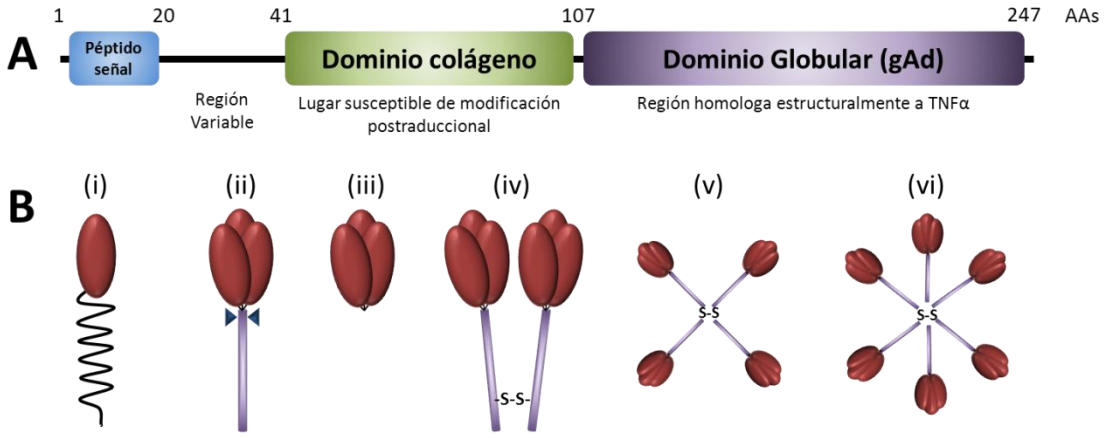


Imagen 8: Estructura del monómero y de los homocomplejos de APN. A, representación esquemática de los 4 dominios de la proteína con la numeración de aas correspondiente a cada uno. B, representación de las diferentes estructuras formadas por APN. (i)Monómero, (ii) homotrímero (LMW) donde se muestra la zona de corte proteolítico (flechas azules) para dar un (iii) homotrímero globular (gAd), (iv) homohexámero (MMW), (v) y (vi) supercomplejos de alto peso molecular (HMW).

Tanto la distribución como la concentración de los multímeros en suero difieren en función del sexo. La adiponectina constituye el 0.01%-0.05% de las proteínas plasmáticas, siendo su concentración muy superior al del resto de citoquinas y hormonas, con valores entre 3-30 μg/mL [116, 172]. Además su liberación muestra un ritmo circadiano con niveles nocturnos muy bajos y un pico a primera hora de la mañana [7]. La secreción de la APN está regulada por diferentes hormonas: mientras que el factor de crecimiento similar a insulina (IGF-1) aumenta sus niveles; el TNFα, los glucocorticoides, los agonistas β adrenérgicos [57]; la prolactina y la somatotropina [137]; y la insulina los disminuye en tejido adiposo [132], tanto en humanos como en ratones.

Alteraciones en los niveles de APN o sus receptores están asociados a diferentes patologías como obesidad [4, 190], diabetes tipo 2 [71], hipertensión [76], síndrome metabólico [164], enfermedad arterial coronaria [98], hiperglucemia e hiperinsulinemia [191] o ayuno [81].

6.2. Receptores de la Adiponectina

En 2003, se clonó cDNA del receptor 1 de APN (AdipoR1) de una librería de músculo esquelético humano que unía la parte globular de la APN. Éste, era una proteína muy conservada desde levaduras hasta humanos, sobre todo en sus 7 dominios trans-membrana. A su vez, se encontró por homología, una proteína 66.7% homóloga a nivel proteico, a la que se le denominó receptor 2 de APN (AdipoR2) [213]. Mientras que AdipoR1 se expresa de forma ubicua, especialmente en músculo esquelético, AdipoR2 lo hace principalmente en hígado [213]. Recientemente, se ha descubierto un tercer receptor. Aunque su estructura es totalmente diferente a AdipoR1 y AdipoR2 [180], la T-caderina que es capaz de unir APN en mioblastos C2C12 [73] aunque no se expresa en hígado, órgano diana de la APN [97, 211].

El extremo C-terminal extracelular de los receptores AdipoR1 y AdipoR2 interacciona con la APN mientras que el dominio N-terminal citoplasmático interacciona con la proteína adaptadora APPL1 [118, 185], que media el incremento en la captación de glucosa y la oxidación de ácidos grasos por la activación de PPARα, p38 MAPK y AMPK [211, 213]. La AMPK, sensor energético celular que se activa cuando la relación AMP/ATP aumenta, actúa como el principal componente en la señalización por APN. Mientras que en hígado, la AMPK inhibe la G6Pasa, inhibiendo la gluconeogénesis y aumentando la sensibilidad a la insulina; en músculo esquelético aumenta la captación de glucosa activando la PI3K y finalmente Akt. A su vez, la activación de la Acetil-CoA carboxilasa (ACC) por AMPK permite la oxidación de ácidos grasos. El adaptador APPL1 también media la activación de la MAPK p38 y la GTPasa Rab5 que

permitirán la translocación a la membrana del canal de glucosa GLUT4, aumentando así los niveles intracelulares de glucosa. A su vez, p38 es capaz de activar al factor de transcripción PPARα, que viajará al núcleo desencadenando la activación de genes implicados en metabolismo lipídico y diferenciación celular (revisado de [4, 29, 81, 118, 211]).

6.3. Acciones metabólicas de la Adiponectina

En los últimos años, un gran número de grupos se han centrado en el estudio de la APN y sus propiedades en diferentes alteraciones metabólicas. Por ejemplo, existe una fuerte correlación negativa entre los niveles de APN circulantes y la resistencia a insulina, tanto en ratones como en humanos [96, 212]. Se han descrito tres mecanismos distintos por los que la APN lleva a cabo sus efectos: **1**. aumento de la transducción de señales del receptor de la insulina [212]; **2**. aumento de la oxidación de ácidos grasos, aumentando la expresión de PPARα [212]; y **3**. aumento de la captación de glucosa, activando la AMPK [211].

La APN también tiene acciones antiaterogénicas. Estudios *in vitro* han demostrado que la APN inhibe la adhesión de monocitos y la expresión de: la E-selectina, la molécula de adhesión vascular-1 (VCAM-1) y la molécula de adhesión intercelular-1 (ICAM-1) [142], además de inhibir la transformación de macrófagos humanos a células espumosas disminuyendo la expresión del receptor scavenger SR-A [143]. Así, la APN puede prevenir la progresión de la enfermedad vascular.

Por todo ello, se han realizado muchos estudios sobre el papel de APN en inflamación. En cultivos de macrófagos, la APN suprime la producción de citoquinas pro-inflamatorias e induce la producción de citoquinas antiinflamatorias [208, 209], lo que indicaría que APN puede promover fenotipos anti-inflamtorios. Por otro lado, ratones APN-KO tienen niveles aumentados de genes M1, como TNFα, IL-6 y MCP-1, y menores niveles de expresión de genes M2 como Arg-1, IL-10 y Mgl-1 en macrófagos peritoneales, respecto a ratones Wt *in vivo* [139].

Sin embargo en otros contextos, se ha propuesto que APN podría promover fenotipos inflamatorios. En cultivos de adipocitos 3T3-L1, las citoquiinas IL-6 y IL-18 inhiben la expresión y secreción de APN *in vitro* [46, 183], al contrario que en macrófagos inducidos por LPS [214]. Además, en células endoteliales, la APN activa la NO sintasa endotelial eNOS (marcador de un fenotipo pro-inflamatorio M1) vía PI3K [131] y la vía de NFκB, promoviendo la inflamación [64]. Además estudios recientes demuestran que la APN ejerce efectos pro-inflamatorios en macrófagos humanos [34] y facilita la actividad fagocítica en macrófagos por su habilidad de unir el sistema Calreticulina/co-receptor (CRT/CD91) a la superficie celular.

Objetivos

Los objetivos planteados en esta Tesis Doctoral han sido:

Identificación de nuevos estímulos en macrófagos, cuya señalización intracelular esté mediada por Cot/tpl-2:

Más concretamente

1. Investigar la implicación de Cot/tpl-2 en la activación de las vías de AKt y mTORC, y su conexión con la regulación de la traducción.

2. Determinar el papel de Cot/tpl-2 en la inflamación estéril inducida por Acetaminofén y DAMPs en modelos *in vivo*.

3. Estudiar el papel de Cot/tpl-2 en el control del estado de activación de los macrófagos tras la estimulación con DAMPs y Adiponectina.

4. Analizar la implicación de Cot/tpl-2 en la expresión génica en macrófagos en respuesta a Adiponectina.

Materiales y Métodos
Resultados

Capítulo I

Cot/tpl-2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor-activated macrophages

López-Peláez, M.; Fugagalli, S.; Sanz, C.; Herrero, C.; Guerra, S.; Fernandez, M.; Alemany, S.

Molecular biology of the Cell (23) 2982-2992 (2012)

En este trabajo se demuestra que el eje Cot/tpl-2--MKK1/2--Erk1/2 modula, a varios niveles, la señal de activación que se transduce tras la activación de PI3K en macrófagos estimulados con LPS. Como consecuencia de esta regulación la actividad Cot/tpl-2 modula la disociación del complejo 4E-BP1--eIF4E, paso clave en el control del inicio de la traducción de mRNAs Cap-dependientes. Así, en este trabajo se demuestra que Cot/tpl-2 regula el reclutamiento de polisomas a los 5' TOP mRNAs, eEF1α y eEF2, así como a los mediadores inflamatorios TNFα, IL-6 y KC. Además, Cot/tpl-2 regula la vida media de los mRNA de estos mediadores inflamatorios.

En macrófagos, tras la estimulación del receptor TLR4 con LPS, Cot/tpl-2 activa Erk1/2. La estimulación del TLR4 también conlleva la activación de PI3K, que activa a PDK1 y esta fosforila a su vez a Akt en T308. La activación completa de Akt se produce cuando se fosforila en un segundo residuo, en S473, llevado a cabo por mTORC2. Otra diana de mTORC2 es SGK1 a la que fosforila en S422. La deficiencia de Cot/tpl-2 en macrófagos estimulados con LPS no altera la fosforilación de Akt en T308, indicando que Cot/tpl-2 no altera la activación ni de PI3K, ni de PDK1. Sin embargo tanto la fosforilación de Akt en S473 como la fosforilación de SGK1 en S422, ambos sustratos de mTORC2, está disminuida en macrófagos Cot/tpl-2 KO en comparación con los niveles observados en macrófagos Wt tras la estimulación con LPS. Todos estos datos indican que Cot/tpl-2 no modula la activación de PDK1 por PI3K, pero si la activación del complejo mTORC2. Mientras que la fosforilación de Akt en T308 es suficiente para fosforilar TSC2 en S939 e inhibir al complejo TSC2-TSC1, Akt debe estar fosforilada en ambos residuos para poder fosforilar FOXO1 en T24. En concordancia con estos datos, en este trabajo también demostramos que la deficiente expresión de Cot/tpl-2 en macrófagos estimulados con LPS reduce la fosforilación de FOXO1 en T24, pero no de TSC2 en S939.

Por otra parte, la quinasa RSK, cuya activación ha sido descrita como dependiente de Erk1/2, tiene como sustratos a distintos componentes de la maquinaria de traducción entre los que se encuentran TSC2, S6K1, S6 y eIF2k. TSC2-TSC1 vía mTORC1 regula la activación de S6K1. La deficiente activación en macrófagos Cot/tpl-2 KO de RSK reduce la fosforilación de sus sustratos TSC2, S6K1, S6 y eIF2k, componentes de la maquinaria de traducción. A su vez, la liberación de 4E-BP1 por mTORC1 del complejo 4E-BP1--eIF4E es el paso limitante en la formación del complejo activo y el inicio de la síntesis proteica. Cot/tpl-2 modula la actividad de mTORC1, como demuestra la falta de fosforilación de 4E-BP1 en macrófagos deficientes en Cot/tpl-2. Todos estos resultados son debidos a la falta de actividad y no de la proteína como se demuestra al utilizar macrófagos que contienen una versión inactiva de Cot/tpl-2. Para comprobar que todo este sistema está siendo activado a través de Cot/tpl-2 por Erk1/2, la

adición de un inhibidor específico para éste redujo significativamente la fosforilación de S6, eEF2k y 4E-BP1 en macrófagos Wt. Cot/tpl-2 regula la fosforilación de proteínas claves en la traducción como RSK, eIF4E, eEF2k, S6 y 4E-BP1 no solo tras la estimulación del TLR4 con LPS, sino también tras la activación de TLR2/6 y de TLR3, mediante la estimulación con Zymosan y Poli I:C respectivamente. Por el contrario, macrófagos Wt y Cot/tpl-2 KO presentan niveles de fosforilación de 4E-BP1 similares tras la estimulación con IL-10.

En este trabajo también se demuestra, mediante ensayos de "pull-down" del complejo 4E-BP1--eIF4E, que la deficiente fosforilación de 4E-BP1 en macrófagos Cot/tpl-2 KO no permite la liberación de eIF4E del complejo 4E-BP1--eIF4E tras la estimulación con LPS. Dicha disociación es clave para el inicio de la traducción de los mRNAs Cap-dependientes. Además, la medida de la traducción Cap-dependiente y Cap-independiente en macrófagos Wt y Cot/tpl-2 KO tras la transfección de un plásmido bicistrónico con dos inicios de traducción, uno Cap-dependiente y otro Cap-independiente, y la posterior estimulación con LPS, demuestra que la traducción Cap-dependiente está drásticamente disminuida en macrófagos Cot/tpl-2 KO mientras que la traducción Cap-independiente es similar en macrófagos Wt y Cot/tpl-2 KO.

Posteriormente, y para comprobar que Cot/tpl-2 controla la traducción de mRNas en macrófagos estimulados o no con LPS, se separaron mediante gradientes de sacarosa distintas fracciones de RNA según su tasa de traducción. Tras la estimulación de macrófagos con LPS, se observó un incremento del nº de polisomas unidos al mRNA de eEF1α y eEF2, dos mensajeros incluidos en los 5' TOP mRNAs. Este incremento del nº de polisomas unidos al mRNA es significativamente mayor en macrófagos Wt que en macrófagos Cot/tpl-2 KO y la inhibición de Erk1/2 mimetiza la deficiencia de Cot/tpl-2. Por otro lado, los niveles de traducción de la β-actina, se mantienen constantes en todas las distintas condiciones examinadas.

El análisis de la tasa de traducción de los mediadores inflamatorios TNFα, IL-6 y KC en macrófagos Wt y Cot/tpl-2 KO estimulados con LPS indica que Cot/tpl-2 también promueve el reclutamiento de polisomas a estos mRNAs. Además en ensayos realizados en macrófagos estimulados con LPS en presencia y ausencia de Actinomicina D indican que, independientemente de la regulación de la tasa de traducción, Cot/tpl-2 también modula la vida media de los mRNAs de TNFα, IL-6 y KC.

El doctorando llevó a cabo la generación de macrófagos derivados de médula ósea, ensayos de western blot presentados en la figura 1 y 3, demostrando que LPS, Poli I:C y Zymosan fosforilan eIF4E, eEF2k, S6 vía Cot/tpl-2. Además ha realizado los ensayos de vida media de los mRNAs de TNFα, IL-6 y KC en macrófagos Wt presentado en la figura 7.

Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor–activated macrophages

Marta López-Pelaéz[a], Stefano Fumagalli[b], Carlos Sanz[a], Clara Herrero[a], Susana Guerra[c], Margarita Fernandez[a], and Susana Alemany[a]

[a]Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas, 28029 Madrid, Spain; [b]Institut National de la Santé et de la Recherche Médicale U845, Necker Medical School, Université Paris Descartes, 75015 Paris, France; [c]Department of Preventive Medicine and Public Health, Universidad Autónoma de Madrid, 28029 Madrid, Spain

ABSTRACT **Cot/tpl2 is the only MAP3K that activates MKK1/2-Erk1/2 in Toll-like receptor–activated macrophages. Here we show that Cot/tpl2 regulates RSK, S6 ribosomal protein, and 4E-BP phosphorylation after stimulation of bone marrow–derived macrophages with lipopolysaccharide (LPS), poly I:C, or zymosan. The dissociation of the 4E-BP–eIF4E complex, a key event in the cap-dependent mRNA translation initiation, is dramatically reduced in LPS-stimulated Cot/tpl2-knockout (KO) macrophages versus LPS-stimulated wild-type (Wt) macrophages. Accordingly, after LPS activation, increased cap-dependent translation is observed in Wt macrophages but not in Cot/tpl2 KO macrophages. In agreement with these data, Cot/tpl2 increases the polysomal recruitment of the 5′TOP eEF1α and eEF2 mRNAs, as well as of inflammatory mediator gene–encoding mRNAs, such as tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and KC in LPS-stimulated macrophages. In addition, Cot/tpl2 deficiency also reduces total TNFα, IL-6, and KC mRNA expression in LPS-stimulated macrophages, which is concomitant with a decrease in their mRNA half-lives. Macrophages require rapid fine control of translation to provide an accurate and not self-damaging response to host infection, and our data show that Cot/tpl2 controls inflammatory mediator gene–encoding mRNA translation in Toll-like receptor–activated macrophages.**

Monitoring Editor
Thomas M. Magin
University of Leipzig

Received: Feb 22, 2012
Revised: May 29, 2012
Accepted: May 30, 2012

INTRODUCTION

Translational control of gene expression is an important regulatory step in which the amount of protein to be produced of an already transcribed mRNA is modulated. Translation of mRNAs is subject to tight control by cells, providing a rapid and adequate response to external cell stimuli. In addition, cells have also developed mechanisms to control the stability of mRNAs (Chen and Shyu, 1995; Anderson, 2008; Guo *et al.*, 2010). A key limiting step of translation is the initiation step, during which the small ribosome subunit is recruited to the 5′ untranslated region (UTR) of the cap-mRNA and scans toward the start codon (reviewed in Gingras *et al.*, 1999; Sachs and Varani, 2000; Hellen and Sarnow, 2001). In certain transcripts and under defined conditions, the cap is bypassed by use of an internal ribosome entry site (IRES), which is located in the 5′UTR of transcripts (Pestova *et al.*, 2001).

Cap-dependent translation is facilitated by the recognition of the mRNA 5′m^{7}GpppN cap structure by eIF4F, which includes the cap-binding subunit, eIF4E. The assembly of the active eIF4F is blocked by the reversible association of eIF4E with the translation repressors 4E-binding proteins (4E-BPs), in which the release of 4E-BP is the key event in the control of cap-dependent mRNA translation initiation. Dissociation of the eIF4E–4E-BP complex is achieved by 4E-BP phosphorylation, controlled by the mammalian/mechanistic target of rapamycin complex 1 (mTORC1; reviewed in Gingras *et al.*, 1999;

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-02-0135) on June 6, 2012.

Address correspondence to: Susana Alemany (salemany@iib.uam.es).

Abbreviations used: BMDM, bone marrow–derived macrophages; 4E-BP, 4E-binding protein; eEF2k, elongation factor 2 kinase; FBS, fetal bovine serum; HMP, high–molecular weight polysomes; IL-6, interleukin-6; IRES, internal ribosome entry site; KD, kinase dead; KO, knockout; LMP, low–molecular weight polysomes; LPS, lipopolysaccharide; m^{7}GTP, 7-methyl GTP; mTORC, mammalian/mechanistic target of rapamycin complex; NP, nonpolysomal; TLR, toll-like receptor; TNF α, tumor necrosis factor α; 5′TOP, 5′terminal oligopirimidine tract; qRT-PCR, quantitative real-time PCR; UTR, untranslated region; WT, wild type.

Pestova *et al.*, 2001). However, cap-dependent translation of different mRNAs is not equally sensitive to mTORC1 activity, with 5′TOP mRNAs, as well as mRNAs with highly structured 5′UTR, being very sensitive to mTORC1 activity (Topisirovic *et al.*, 2011; Huo *et al.*, 2012; Thoreen *et al.*, 2012). mTORC1 also phosphorylates/activates S6K1 (p70 S6k), which is responsible for the phosphorylation of the ribosomal protein S6. S6K1 also upregulates peptide elongation by phosphorylating and consequently inhibiting elongation factor 2 kinase (eEF2k), which phosphorylates and represses eEF2 (Wang *et al.*, 2001). PI3K signaling connected via Akt, by direct phosphorylation of TSC2, can block the TSC1/TSC2 complex inhibitory action on mTORC1 activity (reviewed in Gingras *et al.*, 1999; Sachs and Varani, 2000; Hellen and Sarnow, 2001). Indeed, the PI3K intracellular pathway controls mRNA translation (Laplante and Sabatini, 2009), including the translation of 5′TOP mRNAs, which contain a 5′-terminal oligopyrimidine tract (5′TOP) and encode components of the translational machinery (Jefferies *et al.*, 1997; Meyuhas, 2000; Tang *et al.*, 2001; Patursky-Polischuk *et al.*, 2009). On the other hand, the activity of mTORC1 and of its downstream effectors is also enhanced by the kinase RSK (Anjum and Blenis, 2008; Pearce *et al.*, 2010). Activation of the RAS-RAF-MKK1/2-Erk1/2 pathway triggers RSK phosphorylation/activation, which subsequently phosphorylates TSC2 (Roux *et al.*, 2004). RSK also phosphorylates the subunit of mTORC1, Raptor (Carriere *et al.*, 2008), and S6 (Roux *et al.*, 2007), as well as eEF2k (Bain *et al.*, 2007). Indeed, both RAF and PI3K pathways stimulate mTORC1 signaling (Anjum and Blenis, 2008; Pearce *et al.*, 2010).

Activation of PI3K occurs by a broad array of different stimuli in a variety of different cell types. In innate immune cells such as macrophages, PI3K activation, among other intracellular signaling pathways, occurs upon activation of different Toll-like receptors (TLRs; reviewed in Fukao and Koyasu, 2003; Chaurasia *et al.*, 2010). Receptors of this family sense infection and are stimulated by different pathogen-associated molecular patterns. Activation of the different intracellular pathways upon TLRs stimulation orchestrates the first line of resistance against infection, triggering the production of cytokines and chemokines critical for host defense (Kawai and Akira, 2011). All TLRs, except TLR3, recruit the adaptor MyD88, which transduces the intracellular signal to activate the kinase TAK1. Consequently, TAK1 activates the p38α and JNK MAP kinase pathways, as well as the canonical IKKs, IKKα and IKKβ. TLR3 and TLR4 recruit the TRIF adaptor, which specifically activates the noncanonical IKKs but also IKKα and IKKβ (reviewed in Akira and Takeda, 2004; O'Neill and Bowie, 2007). Activated IKKβ phosphorylates IκB and p105 NF-κB, targeting IκB to degradation and p105 NF-κB to partial proteolysis. In resting cells, Cot/tpl2 (MAP3K8) forms a stable and inactive complex with p105 NF-κB and ABIN2, protecting Cot/tpl2 from its degradation. The partial proteolysis of p105 NF-κB to p50 NF-κB releases Cot/tpl2 from the complex (reviewed in Gantke *et al.*, 2011; Vougioukalaki *et al.*, 2011). On TLR stimulation, dissociated Cot/tpl2 with an adequate phosphorylation state fully activates MKK1/2 and consequently Erk1/2 (Dumitru *et al.*, 2000; Caivano *et al.*, 2003; Cho and Tsichlis, 2005; Banerjee *et al.*, 2006; Stafford *et al.*, 2006) before being rapidly degraded through the proteasome pathway (Gandara *et al.*, 2003; Waterfield *et al.*, 2003). Cot/tpl2 is the only MAP3K that activates the Erk1/2 pathway under these cell stimulation conditions and fulfils a role in innate immunity and inflammatory hypernociception that cannot be substituted for by any other protein (Cohen, 2009; Soria-Castro *et al.*, 2010; Gantke *et al.*, 2011; Vougioukalaki *et al.*, 2011). Cot/tpl2 participates in the production of interleukin-1β and represses interferon-β transcription (Kaiser *et al.*, 2009; Mielke *et al.*, 2009; Lopez-Pelaez *et al.*, 2011). In addition, Cot/tpl2 has a critical role in the production of tumor necrosis factor α (TNFα) during inflammatory responses; it regulates the processing of the pre-TNFα protein before its secretion (Rousseau *et al.*, 2008), modulates the nuclear export of TNFα mRNA (Dumitru *et al.*, 2000), and also enhances TNFα gene transcription (Ballester *et al.*, 1998).

In the context of innate immunity, and in addition to the controlled transcriptional activation, the control at the level of translation of cytokine and chemokine mRNAs plays an essential role in the required rapid and accurate response against infection without damage of the host cells (reviewed in Anderson, 2008, 2010; Hao and Baltimore, 2009).

Here we show that Cot/tpl2 controls mTORC1-dependent mRNA translation in TLR-activated macrophages. Cot/tpl2, via Erk1/2, regulates the dissociation of the 4E-BP–eIF4E complex and the mRNA polysomal recruitment of 5′TOP mRNAs. Cot/tpl2 also controls the recruitment onto translating polysomes of mRNAs that encode proteins involved in the innate immune response such as TNFα, IL-6, and KC. In addition, Cot/tpl2 also increases the half-life of these mRNAs, affecting their total mRNA expression levels.

RESULTS

Cot/tpl2 controls, in an Erk1/2-dependent manner, 4E-BP1 phosphorylation in TLR-stimulated macrophages

Cot/tpl2 mediates Erk1/2 activation in the macrophagic cell line RAW, peritoneal macrophages, and bone marrow–derived macrophages (BMDM) upon engagement of lipopolysaccharide (LPS) to TLR4 (Dumitru *et al.*, 2000; Caivano *et al.*, 2003; Figure 1A). Subsequently, activated Cot/tpl2 is rapidly degraded through the proteasome pathway (Gandara *et al.*, 2003; Waterfield *et al.*, 2003). In addition, in LPS-activated BMDM, Cot/tpl2 controls the phosphorylation of Akt on S473 (Lopez-Pelaez *et al.*, 2011; Figure 1A), a residue phosphorylated by mTORC2 (Sarbassov *et al.*, 2005). The phosphorylation of SGK1 on S422, which is also dependent on mTORC2 activity (Garcia-Martinez and Alessi, 2008), was also diminished in LPS-stimulated Cot/tpl2-knockout (KO) BMDM compared with LPS-stimulated wild-type (Wt) BMDM (Figure 1A). However, the loss of Cot/tpl2 expression did not affect the phosphorylation of T308 in the activation loop of Akt by PDK1. These data indicate that Cot/tpl2 deficiency does not affect the activation of PDK1 by PI3K in LPS-treated macrophages but suggest deficient mTORC2 activation in LPS-treated Cot/tpl2 KO BMDM versus their Wt counterparts. Akt needs to be phosphorylated on both residues S473 and T308 to be fully active and to phosphorylate FOXO1 on T24; however, phosphorylation of Akt on T308 is sufficient to phosphorylate TSC2 on S939 (Guertin *et al.*, 2006). Accordingly, T24 FOXO1 phosphorylation was clearly reduced in Cot/tpl2 KO BMDM versus Wt BMDM upon LPS stimulation, whereas S939 TSC2 phosphorylation was only slightly reduced, just by 20% after 90 min of LPS stimulation (Figure 1A).

Compared to their Wt counterparts, LPS-stimulated Cot/tpl2 KO BMDM showed a statistically significant reduction of T573 RSK phosphorylation (Figure 1B), explaining the reduced phosphorylation in the same setting of its substrate TSC2 in residue S1798 and of the downstream effectors S6K1, S6, and eEF2k (Figure 1B). The phosphorylation of 4E-BP by mTORC1 is the key signal that triggers the dissociation of eIF4E from the inactive 4E-BP–eIF4E complex, allowing eIF4E to form the active eIF4F complex. Active eIF4F facilitates the initiation of translation by its binding to the m^7GpppN cap site located at the 5′-terminus of all mRNAs (Gingras *et al.*, 1999; Pestova *et al.*, 2001). Decreased phosphorylation of 4E-BP1 in Cot/tpl2 KO BMDM versus their Wt counterparts was already observed in basal conditions, and upon LPS stimulation, the difference in the 4E-BP1 phosphorylation ratio was further increased by Cot/tpl2

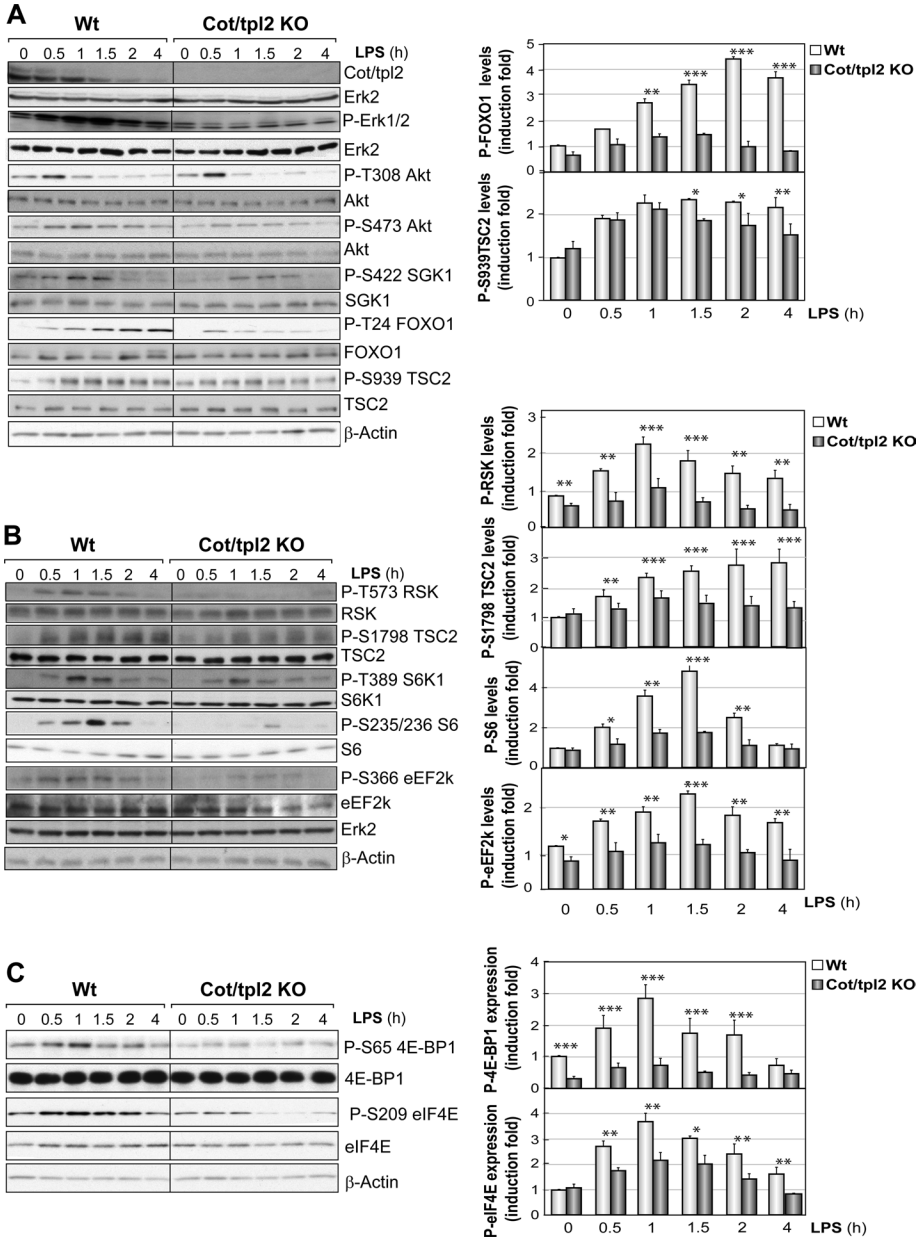


FIGURE 1: Cot/tpl2 governs the phosphorylation state of proteins involved in cap-dependent translation in LPS-stimulated BMDM. (A) Wt and Cot/tpl2 KO BMDM were stimulated with LPS (300 ng/ml), and after the indicated times Cot/tpl2, P-Erk1/2, P-T308 Akt, P-S473 Akt, P-S422 SGK1, P-T24 FOXO1, and P-S939 TSC2 levels were measured by Western blot. Erk2, Akt, SGK1, FOXO1, TSC2, and β-actin levels were determined as a protein loading control. Right, graphs represent the means ± SD from five independent experiments of P-T24 FOXO1 and P-S939 TSC2 fold induction relative to the Wt zero time point, after normalizing values to, respectively, total FOXO1 and total TSC2. (B) Cell extracts obtained as described in A were subjected to Western blot analysis using antibodies against the following phosphoproteins: P-T573 RSK, P-S1798 TSC2, P-T389 S6K1, P-S235/236 S6, and P-S366 eEF2k.The antibodies against total proteins used were RSK, TSC2, S6K1, S6, eEF2k, Erk2, and β-actin. The graphs represent the means ± SD from at least four independent experiments of P-T573 RSK, P-S1798 TSC2, P-S235/236 S6, and P-S366 eEF2k fold induction relative to the Wt zero time point, after normalizing values to, respectively, total RSK, TSC2, S6, and eEF2k. (C) Western blots of cell extracts obtained as described in A were probed against P-S65 4E-BP1, P-S209 eIF4E, 4E-BP1, eIF4E, and β-actin. Right, graphs represent the means ± SD from five independent experiments of P-S65 4E-BP1 and P-S209 eIF4E fold induction relative to the Wt zero time point, after normalizing values to, respectively, total 4E-BP1 and total eIF4E (A, B). Quantification of the induction fold of P-Erk1/2, P-T308 Akt, P-S473 Akt, P-S422 SGK1, and P-T389 S6K1 levels is shown in Supplemental Table S1.

deficiency (Figure 1C). Of note, total 4E-BP1 expression levels were very similar in both Wt and Cot/tpl2 KO BMDM. On the other hand, the phosphorylation of eIF4E on S209 by MNK, a p38α- and Erk1/2-dependent kinase (Marzec *et al.*, 2011), was also diminished by Cot/tpl2 deficiency in LPS-stimulated BMDM.

To examine whether the observed impaired phosphorylation of proteins involved in the initiation of the cap-dependent mRNA translation by Cot/tpl2 deficiency was just due to the lack of Cot/tpl2 activity or to the knockout of Cot/tpl2 protein expression, we used Cot/tpl2 kinase-dead (KD) BMDMs, which express Cot/tpl2 with a point mutation in which the lysine responsible for ATP binding (Lys-167) is replaced by Arg and thereby does not have kinase activity. Indeed, the Cot/tpl2 catalytically inactive protein was not degraded after LPS stimulation, indicating that Cot/tpl2 activity participates in a negative feedback loop ending in its own degradation (Figure 2A). Because S235/236 S6 and S366 eEF2k phosphorylation, as well as the phosphorylation S65 4E-BP1 and S209 eIF4E was also diminished in LPS-stimulated Cot/tpl2 KD BMDM as compared with their Wt counterparts (Figure 2A), we conclude that is just the activity of Cot/tpl2 that is essential in the activation of proteins involved in the cap-dependent mRNA translation. In addition, LPS-stimulated Wt BMDM preincubated with PD 0325901, a specific MKK1/2 inhibitor (Bain *et al.*, 2007), also showed impaired phosphorylation of S6, eEF2k, eIF4E, and 4E-BP1 compared with Wt BMDM after LPS stimulation in the absence of the inhibitor (Figure 2B). Furthermore, pretreatment with UO126, another MKK1/2 inhibitor, or with rapamycin, an inhibitor of mTORC1 activity, also reduced the phosphorylation state of 4E-BP1 in LPS-stimulated Wt BMDM (Figure 2C). Taken together, these data indicate that the capacity of Cot/tpl2 to control Erk1/2 phosphorylation in LPS-activated BMDM is essential for the modulation of the phosphorylation/activation state of proteins involved in the mRNA translation process in LPS-activated macrophages.

LPS signals through both TRIF and MyD88 adaptors after TLR4 activation (Akira and Takeda, 2004; O'Neill and Bowie, 2007). In macrophages with low levels of the receptor dectin-1, zymosan stimulates TLR2/6 and signals through the MyD88 adaptor (Gantner *et al.*, 2003), whereas poly I:C activates TLR3, a receptor that is incapable of recruiting MyD88 and signals through TRIF (O'Neill and Bowie,

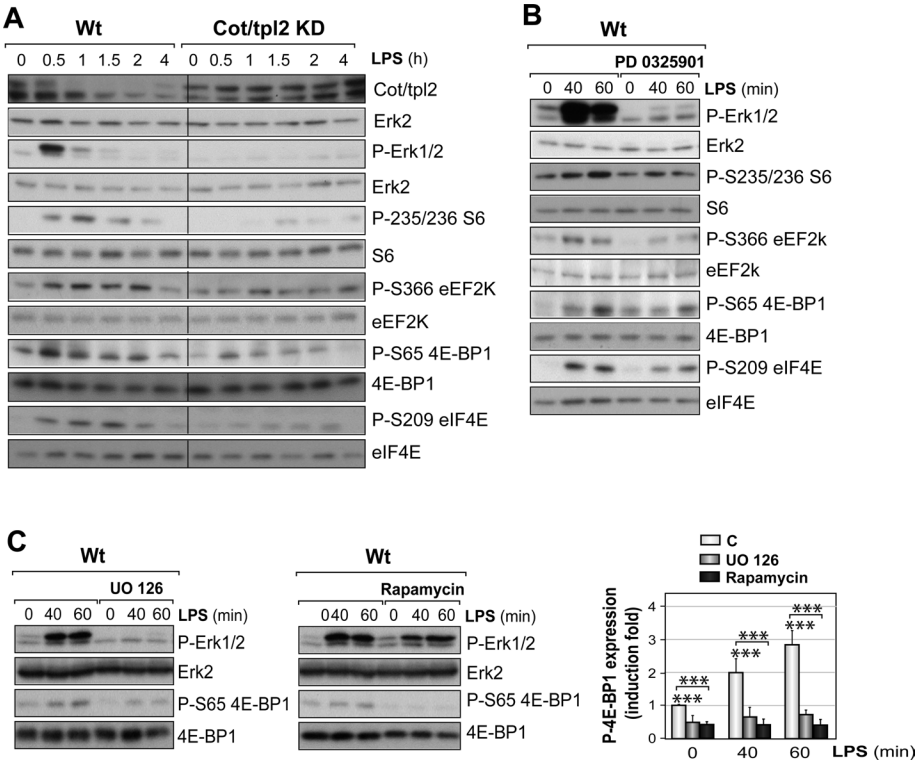


FIGURE 2: Erk1/2-dependent phosphorylation of P-S235/236 S6, P-S366 eEF2k, P-S65 4E-BP1, and P-S209 eIF4E in LPS-stimulated BMDM. (A) Wt and Cot/tpl2 KD BMDM were stimulated for different lengths of time with LPS (300 ng/ml), and the extracts obtained were analyzed by Western blots probed with the phospho antibodies against P-Erk1/2, P-S235/236 S6, P-S366 eEF2k, and P-S65 4E-BP1 and with antibodies against the total protein of Cot/tpl2, Erk2, S6, eEF2k, 4E-BP1, and eIF4E as loading controls. (B) Wt BMDMs were preincubated or not with the MKK1/2 inhibitor PD 0325901 (0.5 μM) for 60 min before stimulation with LPS (300 ng/ml) for the indicated times, after which the levels of P-Erk1/2, P-S235/236 S6, P-S366 eEF2k, P-S65 4E-BP1, and P-S209 eIF4E were determined by Western blot analysis. As a loading control, membranes were also blotted with the following antibodies: Erk2, S6, eEF2k, 4E-BP1, and eIF4E. (C) Wt BMDM were preincubated or not with the MKK1/2 inhibitor UO 126 (10 μM) or rapamycin (20 nM) for 60 min before stimulation with LPS (300 ng/ml) for the indicated times, after which the levels of P-Erk1/2, P-S65 4E-BP1, Erk2, and 4E-BP1 were determined by Western blot analysis. Graph represents the means ± SD from three independent experiments of P-S65 4E-BP1 fold induction relative to the Wt zero time point, after normalizing values to total 4E-BP1. For A–C, one representative experiment of the three independently performed is shown.

2007). Results obtained in Wt and Cot/tpl2 KO BMDM stimulated with poly I:C or with zymosan and analyzed by Western blot indicated that RSK, eIF4E, eEF2k, S6, and S65 4E-BP1 phosphorylation was also controlled by Cot/tpl2 under these cell stimulation conditions (Figure 3). However, Wt and Cot/tpl2 KO BMDM showed similar levels of S65 4E-BP phosphorylation after 15 and 30 min of stimulation with IL-10 (Supplemental Figure S1).

Cot/tpl2 regulates cap-dependent translation in LPS-activated macrophages

We next decided to evaluate whether the reduced 4E-BP1 phosphorylation observed by Cot/tpl2 deficiency in TLR-activated BMDM correlated with a diminished dissociation of the 4E-BP1–eIF4E complex. To this end, extracts from Wt and Cot/tpl2 KO BMDM nontreated or treated for 1 or 2 h with LPS were incubated with the m^7GpppN cap affinity resin, and the levels pulled-down of eIF4E and 4E-BP1 proteins were subsequently determined by Western blot analysis (Figure 4A). In basal conditions, Cot/tpl2 KO BMDM already showed increased levels of 4E-BP1 bound to eIF4E compared with Wt BMDM. Furthermore, the amount of 4E-BP1 bound to eIF4E decreased in a statistically significant manner upon LPS stimulation of Wt BMDM, but the absence of Cot/tpl2 expression severely affected the release of 4E-BP1 from the 4E-BP1–eIF4E complex in LPS-stimulated BMDM (Figure 4A). These data prompted us to study the cap-dependent and cap-independent translation rates in LPS-stimulated Wt and Cot/tpl2 KO BMDM. To this end, cells were nucleofected with a biscitronic plasmid in which *Renilla* luciferase cDNA can be only translated by cap-dependent scanning mechanism, whereas translation of Firefly luciferase does not happen unless internal initiation occurs at the IRES element (Figure 4B). Similar levels of cap-independent translation were observed in Wt and Cot/tpl2 KO BMDM stimulated or not with LPS. Nevertheless, cap-dependent translation was increased in Wt BMDM compared with Cot/tpl2 KO BMDM in basal conditions, and, as expected, LPS stimulation of Wt BMDM further increased cap-dependent translation in a statistically significant manner . However, this increase was hardly detected in Cot/tpl2 KO BMDM after LPS stimulation, resulting in a statistically significant increase in the cap-dependent/cap-independent translation ratio in Wt BMDM versus Cot/tpl2 KO BMDM (Figure 4B).

Cot/tpl2 controls the translation of the 5′TOP eEF1α and eEF2 mRNAs

BMDM from Wt and Cot/tpl2 KO mice were treated or not with LPS for 3 h, and sucrose gradient was performed to separate nonpolysomal mRNAs and nontranslating free 40S and 60S ribosomal subunits from moderately and from actively translating mRNA polysomes. The nonpolysomal fraction (NP) contained the pool of mRNAs that were not associated with components of the translation machinery or cosedimented with ribosome subunits (monosomes); thus, they were not considered to be translated. The mRNAs contained in the fraction with small polysomes of low molecular weight (LMP) were considered to be translated at moderate levels. The third mRNA fraction contained mRNAs that were associated with polysomes of high molecular weight (HMP), and they were thus considered to be actively translated (Supplemental Figure S2). On 3 h of LPS stimulation of Wt and Cot/tpl2 KO BMDM, there was an increase in the recruitment of the 5′TOP eEF1α mRNA to HMP in detriment to the levels detected in the inactive pool. However, the increase in the recruitment of eEF1α mRNA on the actively translating polysomes was, in a statistically significant way, lower in Cot/tpl2 KO BMDM versus Wt BMDM, as determined by Northern blot analysis. By contrast, β-actin mRNA, which is known to be constitutively translated, was associated mostly with the HMP fraction in stimulated and nonstimulated cells and irrespective of their genotype (Figure 5A). Similar reduction in the eEF1α mRNA polysomal recruitment was observed in LPS-stimulated Wt BMDM in the presence of UO126 compared with Wt BMDM stimulated with LPS in the absence of this inhibitor (Supplemental Figure S3). According to these

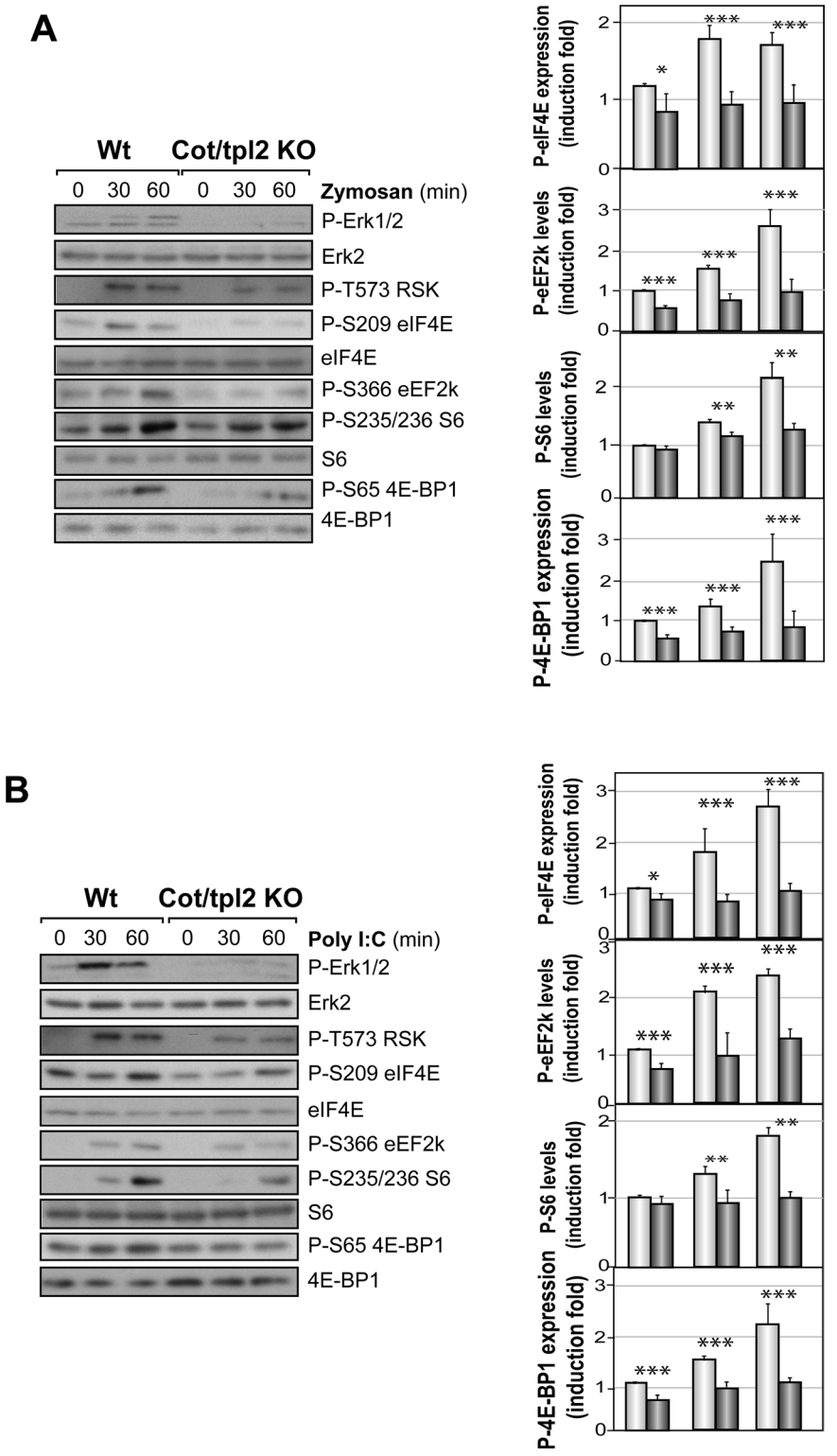


FIGURE 3: P-S65 4E-BP1 phosphorylation in Wt and Cot/tpl2 KO BMDM stimulated with zymosan or poly I:C. Wt and Cot/tpl2 KO BMDM were stimulated with zymosan (10 μg/ml; A) or with poly I:C (50 μg/ml; B) for 30 and 60 min and the expression levels of P-Erk1/2, P-T573 RSK, P-S209 eIF4E, P-S366 eEF2k, P-S235/236 S6, and P-S65 4E-BP1 were determined in Western blots. As a loading control the expression levels of Erk2, eIF4E, S6, and 4E-BP1 were also analyzed. For both A and B, one representative experiment of the three independently performed is shown. Right, graphs represent the means ± SD from five independent experiments of P-S209 eIF4E, eEF2k, P-S235/236 S6, and P-S65 4E-BP1 fold induction relative to the Wt zero time point, after normalizing values to, respectively, total eIF4E, Erk2, S6, and 4E-BP1.

data, Cot/tpl2 deficiency also impaired eEF1α mRNA distribution within polysomal versus nonpolysomal RNA fractions (Supplemental Figure S2) as performed by quantitative real-time PCR (qRT-PCR) analysis (Figure 5B), and again no change in the β-actin distribution was observed in these analysis conditions. To determine whether the effects of Cot/tpl2 were unique for the EF1α mRNA or could be extended to other 5′TOP mRNAs, we also analyzed the recruitment onto polysomes of another 5′TOP mRNA, the eEF2 mRNA. In LPS-activated Wt BMDM this transcript was mainly located in the translated mRNA fraction, but under the same conditions Cot/tpl2 deficiency mainly abolished eEF2 mRNA polysomal recruitment upon LPS stimulation (Figure 5B). Of note, similar levels of total eEF1α, eEF2, or β-actin mRNA were detected in Wt and Cot/tpl2 KO BMDM stimulated or not with LPS (Supplemental Figure S4).

Cot/tpl2 modulates polysomal recruitment of TNFα, IL-6, and KC mRNA in LPS-activated BMDM

We previously showed that in LPS-activated macrophages Cot/tpl2 deficiency triggered a fivefold increase in the IRF1 mRNA levels but only approximately twofold in the IRF1 protein levels respect to Wt BMDM in the same cell conditions (Lopez-Pelaez *et al.*, 2011). The polysomal mRNA distribution of this transcription factor, involved in macrophage activation (reviewed in Tamura *et al.*, 2008), indicated that Cot/tpl2 expression is required to increase the recruitment of IRF1 mRNA to polysomes upon LPS stimulation of BMDM (Supplemental Figure S5). Furthermore, the recovery of IκBα protein levels after TNFα stimulation of mouse embryonic fibroblasts (Das *et al.*, 2005) or upon LPS stimulation of BMDM (Lopez-Pelaez *et al.*, 2011) is diminished in the absence of Cot/tpl2. The similar expression levels of IκBα mRNA after LPS stimulation of Wt and Cot/tpl2 KO BMDM (Supplemental Figure S5) could not explain the previously reported impaired IκBα protein expression; however, Cot/tpl2 deficiency reduced IκBα mRNA levels in the polysomes fraction upon 3 h of LPS stimulation (Supplemental Figure S5), arguing that Cot/tpl2 stimulates the translation of IκBα mRNA. Of interest, IκBβ showed similar protein expression levels in Wt and Cot/tpl2 KO BMDM after LPS stimulation (unpublished data), and Cot/tpl2 did not appear to regulate IκBβ mRNA recruitment to polysomes (Supplemental Figure S5).

Cot/tpl2 controls TNFα production in LPS-activated macrophages (Dumitru *et al.*, 2000; Rousseau *et al.*, 2008; Figure 6A). In addition, an analysis of the TNFα mRNA distribution within the polysomal and nonpolysomal RNA fractions upon LPS stimulation of Wt and Cot/tpl2 KO BMDM indicated that Cot/tpl2 increased TNFα mRNA polysomal recruitment (Figure 6B).

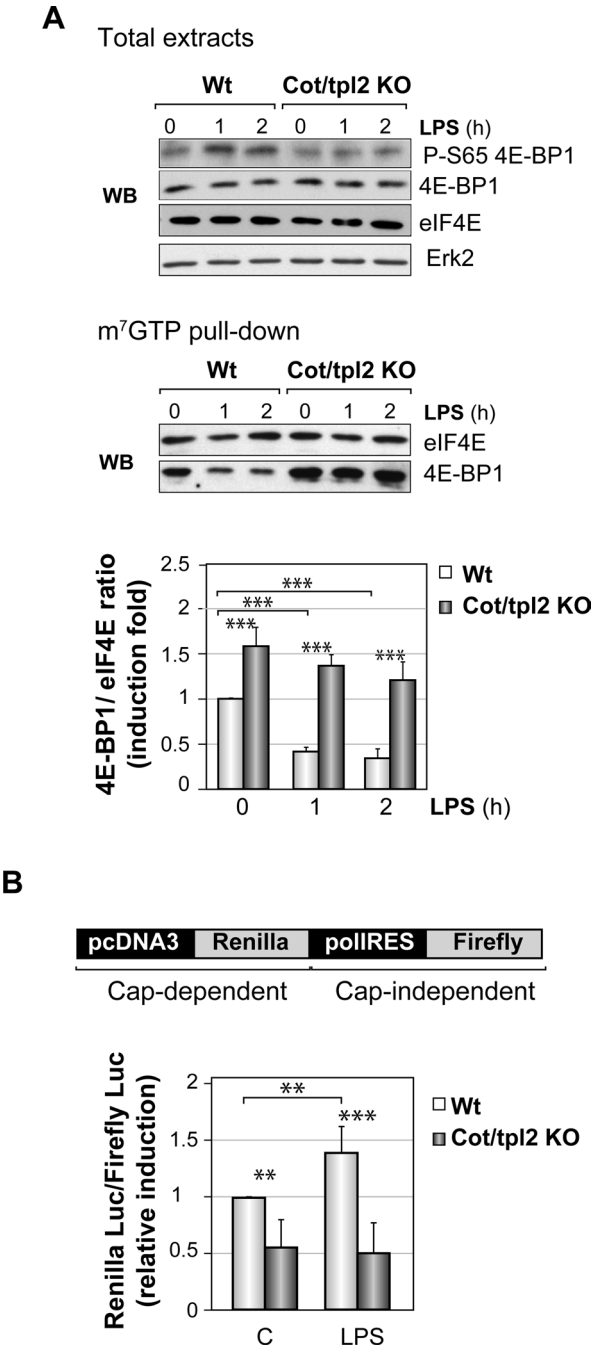


FIGURE 4: Cot/tpl2 promotes dissociation of the 4E-BP1–eIF4E complex and cap-dependent translation. (A) Cell extracts from Wt and Cot/tpl2 KO BMDM stimulated or not for 1 or 2 h with LPS (300 ng/ml) were subjected to m^{7}GTP–Sepharose bead pull-down assays, and the amount of 4E-BP1 bound to eIF4E was analyzed by Western blot. Expression levels of P-S65 4E-BP1, 4E-BP1, eIF4E, and Erk2 in total cell extracts are also shown. Representative experiments of the three independently performed are shown. Graph represents the means ± SD from three independent experiments of the 4E-BP1/eIF4E values, giving the value of 1 to the one obtained in Wt BMDM at zero point time. (B) Wt and Cot/tpl2 KO BMDM were nucleofected as indicated in *Materials and Methods*, with the biscitronic plasmid specified in the figure. Cells were harvested, and *Renilla* and Firefly luciferase luminescence was quantified using a luminometer. Graph represents the means ± SD from three independent experiments of *Renilla*/firefly luciferase values, giving the value of 1 to the one obtained in Wt BMDM at zero time point.

Furthermore, Cot/tpl2 deficiency diminished the levels of other two inflammatory mediators, IL-6 and KC, in the incubation media of BMDM upon LPS stimulation (Figure 6A), and LPS-stimulated Cot/tpl2 KO BMDM showed a severely diminished recruitment of IL-6 and KC mRNAs to polysomes compared with their Wt counterparts (Figure 6B).

Cot/tpl2 increases TNFα, IL-6, and KC mRNA stability in LPS-activated BMDM

In addition to the reduced recruitment of TNFα, KC, and IL-6 mRNA to polysomes, Cot/tpl2 deficiency also reduced total mRNA levels of TNFα, KC, and IL-6 ~3-, 2.5-, and 6.5-fold, respectively, in 3 h LPS-stimulated BMDM (Figure 7A). To determine whether Cot/tpl2 enhances the stability of these mRNAs, Wt and Cot/tpl2 KO BMDM were first treated with LPS for 3 h to induce their expression, and subsequently, to block transcription, actinomycin D was added to the cell culture media and the decay in the expression levels of TNFα, KC, and IL-6 mRNA was examined. In LPS-stimulated BMDM, Cot/tpl2 stabilizes TNFα, KC, and IL-6 mRNA, with an increase in the half-life of ~2.5-fold for TNFα and IL-6 mRNA and ~5-fold for the KC transcript (Figure 7B), indicating that the increase in the total mRNA levels of these cytokines and chemokine by Cot/tpl2 after LPS stimulation could be mainly due to a stabilization of their mRNAs. Of note, the half-life of β-actin mRNA was not affected by Cot/tpl2 deficiency. Taken together, these data indicate that Cot/tpl2 not only controls the translation of inflammatory mediator gene–encoding mRNAs but also controls the mRNA stability of cytokine and chemokine transcripts in the context of the innate immune response generated upon LPS stimulation of macrophages.

DISCUSSION

Cot/tpl2 has emerged as an attractive target to develop new and improved anti-inflammatory drugs (Cohen, 2009; Gaestel *et al.*, 2009) since it plays a role after TLR activation of macrophages that affects the innate immune response (reviewed in Gantke *et al.*, 2011). Here we show a novel function for Cot/tpl2 in controlling the activation state of proteins involved in cap-dependent translation initiation. The PI3K-Akt-TSC2-mTORC1 pathway plays, through their downstream effectors, a key role in the maintenance of translation (Laplante and Sabatini, 2009). However, the fact that in TLR-activated macrophages Cot/tpl2 deficiency does not alter the phosphorylation of Akt on T308 but only on S473 indicates that Cot/tpl2 does not modulate 4E-BP phosphorylation through Akt, since the partial activation of Akt, P-T308 Akt, by PI3K-PDK1 upon TLR stimulation is sufficient to transduce the activating signal to TSC2 (Guertin *et al.*, 2006). On the other hand, the RAS-RAF-MKK1/2-Erk1/2 pathway, by activating the Erk1/2-dependent kinase RSK, also triggers mTORC1 activation (reviewed in Anjum and Blenis, 2008; Pearce *et al.*, 2010). Stimulation of the receptors of the TLR/IL-1R superfamily does not activate any of the RAF proteins, and the activation of the MKK1/2-Erk1/2 pathway is entirely mediated by Cot/tpl2 (Caivano *et al.*, 2003; Cho and Tsichlis, 2005; Banerjee *et al.*, 2006; Rodriguez *et al.*, 2006; Gantke *et al.*, 2011). On the basis of the data shown here, and taking into account the mechanism by which Cot/tpl2 is activated (Rodriguez *et al.*, 2006; Gantke *et al.*, 2011; Vougioukalaki *et al.*, 2011), we can conclude that after TLR activation the TAK1-IKKβ-Cot/tpl2-MKK1/2-Erk1/2 pathway also has RSK as a downstream effector. In addition, Cot/tpl2-dependent activation of RSK links Cot/tpl2, via Erk1/2, with the capacity to control cap-dependent mRNA translation initiation, the key rate-limiting step in the translation process. Nevertheless, we cannot rule out the possibility that Cot/tpl2-MKK1/2-Erk1/2 also inhibits the TSC1-TSC2 complex and consequently increases

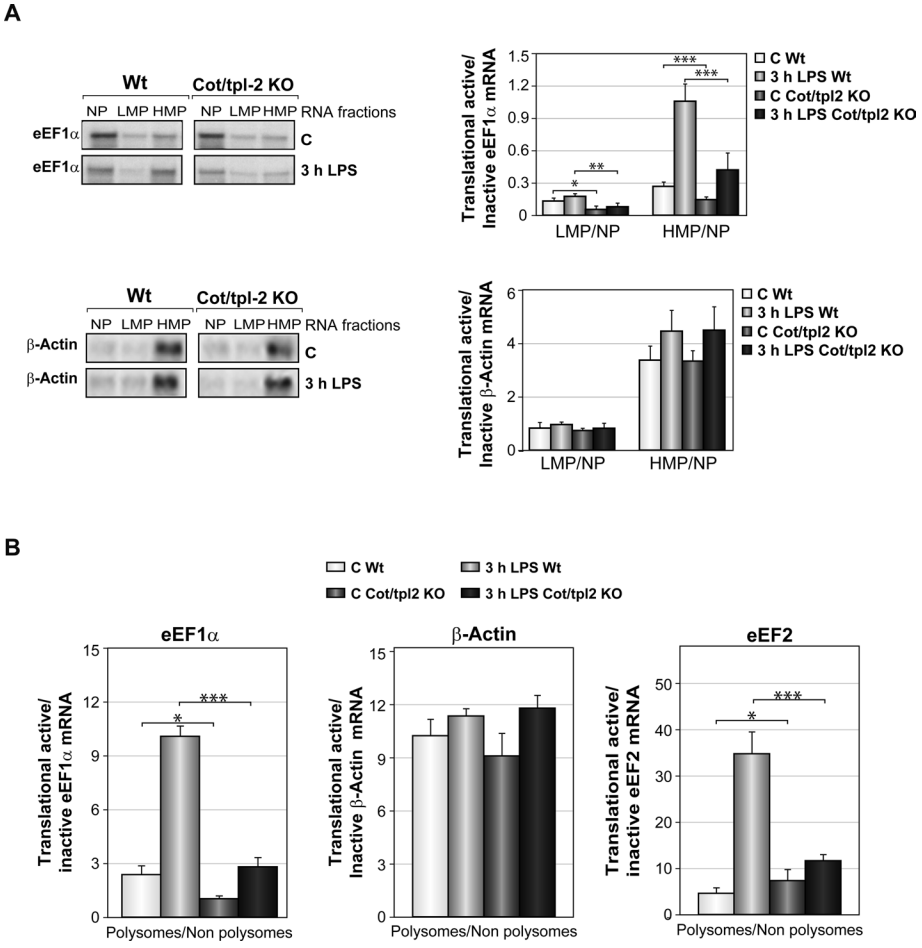


FIGURE 5: Cot/tpl2 regulates polysomal recruitment of eEF1α and eEF2 mRNA in LPS-stimulated BMDM. Cell lysates of Wt and Cot/tpl2 BMDM stimulated for 3 h or not with LPS (300 ng/ml) were subjected to sucrose gradient, and different RNA fractions were pooled. The RNA isolated from the different RNA fractions was subjected to Northern blot or qRT-PCR analysis. (A) Three mRNA fractions from the sucrose gradient were isolated: nontranslated mRNA (nonpolysomal components, consisting of nonpolysomal mRNAs and nontranslating free 40S and 60S ribosomal; NP), moderately translated mRNA (LMP), and actively translated mRNA (HMP). These mRNA fractions were subjected to Northern blot analysis. The eEF1α and β-actin mRNA expression in the NP, LMP, and HMP fractions of nonstimulated and 3-h LPS-stimulated Wt and Cot/tpl2 KO BMDM is shown. Right, graphs represent the means ± SD from five independent experiments of the quantification of the LMP/NP and HMP/NP eEF1α and β-actin mRNA ratios in the different cell conditions. (B) eEF1α, β-actin, and eEF2 mRNA expression in the nonpolysomal and polysomal fractions analyzed by qRT-PCR. Graphs represent the means ± SD from five independent experiments of the quantification of the polysome/nonpolysome mRNA ratio in the different cell conditions.

mTORC1 activity by Erk1/2 direct phosphorylation of TSC2 on S664 (Ma *et al.*, 2005). These data indicate a novel role of Cot/tpl2 in modulating the now evident important role that mTORC1 substrates have in innate immunity development (reviewed in Thomson *et al.*, 2009; Weichhart and Saemann, 2009).

4E-BP phosphorylation by mTORC1 is the key event in the regulation of cap-dependent translation. Here we show that Cot/tpl2-MKK1/2-Erk1/2 triggers 4E-PB1 phosphorylation by activating mTORC1, since not only MKK1/2 inhibitors, but also rapamycin, without inhibiting Erk1/2 activation, inhibits 4E-BP1 phosphorylation in LPS-stimulated Wt BMDM. Dissociation of phosphorylated 4E-BP from the eIF4E–4E-BP complex allows the formation of the active eIF4F that facilitates the initiation of translation by its binding to the m^7GpppN located in the 5′ region of most mRNA sequences (Gingras *et al.*, 1999; Pestova *et al.*, 2001). Accordingly, with the deficient 4E-BP1 phosphorylation in LPS-stimulated Cot/tpl2 KO BMDM versus Wt BMDM, Cot/tpl2 deficiency also prevents in a statistically significant manner the dissociation of the eIF4E–4E-BP complex and diminishes translation of the cap-dependent *Renilla* luciferase. Taken together, these data indicate that Cot/tpl2 controls the activation of the cap–dependent translational machinery in TLR-activated macrophages. Accordingly, mRNA recruitment to polysomes of transcripts encoding proteins that are involved in the innate immune response, such as TNFα, IL-6, and KC, is severely diminished by Cot/tpl2 deficiency.

This newly described capacity of Cot/tpl2 to control the activation state of proteins involved in the mRNA translation process occurs independent of the type of adaptor, MyD88 or TRIF, used by TLRs to transduce the activating signals since it is observed upon both poly I:C and zymosan stimulation of macrophages. Nevertheless, this capacity of Cot/tpl2 is so far a selective and specific event triggered by TLR activation, since Cot/tpl2 has no effect on 4E-BP phosphorylation in BMDM stimulated with IL-10, which uses a receptor distinct from the TLR family (Kotenko *et al.*, 1997), and does not activates Cot/tpl2 (Lopez-Pelaez *et al.*, 2011). In this context, it was recently reported that Cot/tpl2 is also the only MAP3K that activates Erk1/2 in signals initiated by the thrombin-activated, G protein–coupled, receptor protease–activated receptor-1 (Hatziapostolou *et al.*, 2011); thus the possibility that Cot/tpl2 could control cap-dependent translation initiation in other biological processes cannot be excluded.

Here we also describe that Cot/tpl2 regulates the translation of the 5′TOP mRNAs eEF1α and eEF2. The 5′TOP mRNAs contain a 5′-terminal oligopyrimidine tract and encode ribosomal proteins involved in the processing of mRNA, and their translation is very sensitive to mTORC1 activity (Jefferies *et al.*, 1997; Meyuhas, 2000; Tang *et al.*, 2001; Patursky-Polischuk *et al.*, 2009; Thoreen *et al.*, 2012). In this context, it has been shown that sensitivity of mRNA translation to mTORC1 activation varies depending on the mRNA species (Topisirovic *et al.*, 2011; Huo *et al.*, 2012; Thoreen *et al.*, 2012). Indeed, β-actin mRNA translation is barely dependent on mTORC1 activation and 4E-BP1 phosphorylation (Colina *et al.*, 2008; Huo *et al.*, 2012; Thoreen *et al.*, 2012), and our data here show that Cot/tpl2 modulates the downstream effectors of mTORC1 but not polysomal recruitment of β-actin.

Not only does Cot/tpl2 modulate TNFα, KC, and IL-6 mRNA polysomal recruitment in activated macrophages, but it also participates in the expression of their total mRNA levels upon LPS stimulation of macrophages. Total mRNA levels are the result of both the

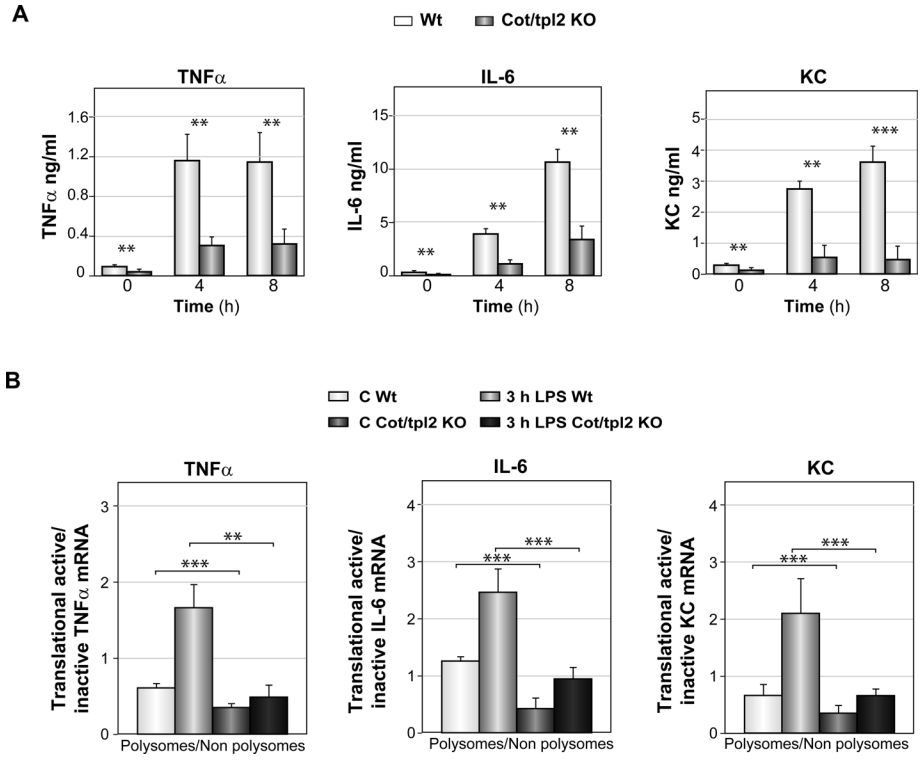


FIGURE 6: Cot/tpl2 controls TNFα, KC, and IL-6 production and polysomal recruitment of their mRNAs in LPS-stimulated BMDM. (A) The concentration of TNFα, IL-6, and KC in the supernatant of Wt and Cot/tpl2 KO BMDM stimulated or not for 3 or 8 h with LPS (300 ng/ml) was determined using a Luminex 100 system (Upstate, Millipore, Billerica, MA), according to the manufacturer's instructions. Graphs show the means ± SD from three independent experiments. (B) TNFα, KC, and IL-6 mRNA expression in the nonpolysomal and polysomal RNA fractions described in Figure 5B was analyzed by qRT-PCR. Graphs represent the means ± SD from five independent experiments of the quantification of the polysome/nonpolysome mRNA ratio in the different cell conditions.

rate of gene transcription and the stability of the mRNA itself. Our data here demonstrate that the increase in the total mRNA levels by Cot/tpl2 after LPS stimulation correlates with the induction in their mRNA stability induced by Cot/tpl2. All three TNFα, IL-6, and KC mRNAs contain AU-rich elements in the 3′ UTR, which are determinant for modulating their half-life in the context of inflammation (reviewed in Anderson, 2008; Mazumder *et al.*, 2010). In this context it was reported that Cot/tpl2 modulates cytoplasmic TNFα mRNA expression by a mechanism that targets the AU-rich element in the 3′ UTR of the TNF-α mRNA upon LPS stimulation (Dumitru *et al.*, 2000). Indeed, MAPKAP-2, as well as MNK1 and 2, downstream kinases of Erk1/2 (Roux and Blenis, 2004), phosphorylate and inhibit hnRP40 activity an AU-rich sequence-specific RNA-binding protein that destabilizes TNFα mRNA (Rousseau *et al.*, 2002; Buxade *et al.*, 2005).

Translational control offers a strategic advantage because the use of the preexisting mRNAs bypasses lengthy nuclear control mechanisms (e.g., transcription, splicing, and transport). At the same time, it provides reversibility through modifications of regulatory intermediates, mainly via reversible phosphorylation, and provides macrophages with required rapid and accurate response capacity. Innate immune system cells are the first line of defense against pathogen infection, and fine control of the translation levels of inflammatory mediators by these cells is a crucial requirement for the proper development of innate immune response. Indeed, the host response against infection is a double-edged sword, as an uncontrolled response can exacerbate tissue damage, and the control of inflammatory mediators at the level of translation has emerged as a key step in adjusting the magnitude of the response (reviewed in Anderson, 2008; Thomson *et al.*, 2009; Mazumder *et al.*, 2010).

Cot/tpl2 has emerged as an interesting new anti-inflammatory target by its capacity to activate Erk1/2, without involving the RAF proteins, during the innate immune response. Here we show that Cot/tpl2 controls mRNA stability and cap-dependent mRNA translation in the context of the innate immune response generated upon the stimulation of macrophages by LPS, modulating at the posttranscriptional level the production of inflammatory mediators involved in the host response.

MATERIALS AND METHODS

Mice, BMDM preparation, and stimulation

C57BL/6J wild type and C57BL/6J Cot/tpl2 KO animals were produced from heterozygous mice (Cot/tpl2 $KO^{+/-}$ × Cot/tpl2 $KO^{+/-}$), and C57BL/6J Cot/tpl2 KD animals were produced from heterozygous C57BL/6J Cot/tpl2 $KD^{+/-}$ mice. All animals received care according to methods approved under institutional guidelines for the care and use of laboratory animals in research. BMDM were obtained as previously described (Lopez-Pelaez *et al.*, 2011), and cells were then stimulated with LPS (*Salmonella typhimurium*; L726; Sigma-Aldrich, St. Louis, MO), poly I:C (Invitrogen, Carlsbad, CA), zymosan (Invitrogen), or IL-10 (PeproTech, Rocky Hill, NJ). Actinomycin D was purchased from Sigma-Aldrich and UO 126 from Tocris Bioscience (Ellisville, MO). PD 0325901 and rapamycin were gifts from, respectively, Philip Cohen (Dundee, Scotland) and Victor Calvo (Madrid, Spain).

Western blot analysis

Cell extracts were examined by Western blot as previously described (Rodriguez *et al.*, 2008), using primary antibodies raised against the following proteins: from Santa Cruz Biotechnology (Santa Cruz, CA), Cot/tpl2, Erk2, P-T389 S6K1, RSK, S6K1, and SGK1; from Cell Signaling Beverly, MA), P-T202/Y204 Erk1/2, P-S473 Akt, P-T308 Akt, Akt, P-T24 FOXO1, FOXO1, P-S939 TSC2, TSC2, β-actin, P-T573 RSK, P-S235/236 S6, P-S366 eEF2k, eEF2k, P-S65 4E-BP1, 4E-BP1, P-S209 eIF4E, and eIF4E; and from Pierce Thermo Fisher Scientific (Rockford, IL), P-S1798 TSC2. Western blot analysis using P-S422 SGK1 (sc-16745R; Santa Cruz Biotechnology) was performed as described previously (Garcia-Martinez and Alessi, 2008). The secondary antibodies used were raised against rabbit (Cell Signaling), goat (Dako, Glostrup, Denmark), and mouse (Amersham, GE Healthcare Bio-Sciences, Piscataway, NJ).

7-Methyl-GTP pull-down assays

BMDM were washed twice in ice-cold phosphate-buffered saline, lysed in lysis/binding buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 0.2% NP-40, 1 U/ml

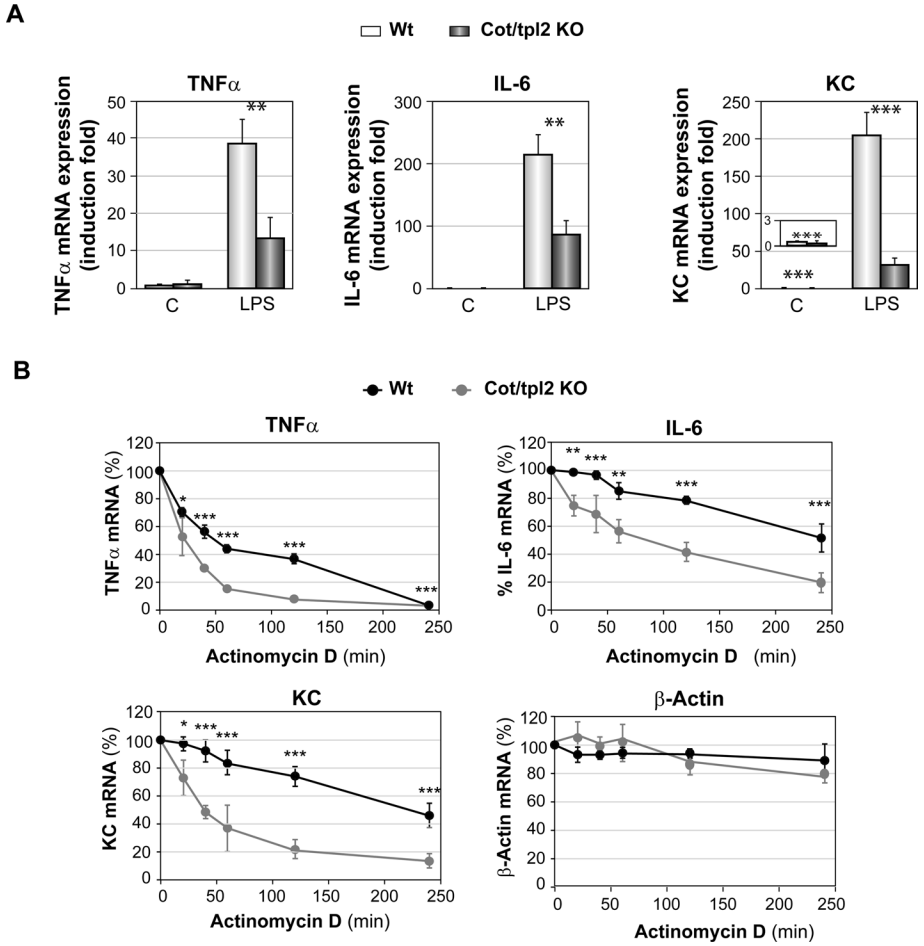


FIGURE 7: Cot/tpl2 controls TNFα, KC, and IL-6 mRNA stability in LPS-stimulated BMDM. (A) Total RNA from Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h with LPS (300 ng/ml) was extracted, and TNFα, KC, and IL-6 mRNA expression was determined by qRT-PCR analysis. TNFα, KC, and IL-6 expression levels were normalized to levels of 18Sr mRNA expression in each assay. Graphs show the mean ± SD from five independent experiments, giving the value of 1 to the one obtained in control Wt BMDM. (B) The stability of TNFα, KC, IL-6, and β-actin mRNA in 3-h LPS-stimulated Wt and Cot/tpl2 KO BMDM was determined. Wt and Cot/tpl2 KO BMDM were stimulated for 3 h with LPS (300 ng/ml), and then actinomycin D (5 μg/ml) was added. At the indicated times cells were lysed, and subsequently the TNFα, IL-6, KC, and β-actin mRNA levels were analyzed by qRT-PCR and normalized by the expression of 18Sr mRNA. Graphs shown the means ± SD from three independent experiments, giving the value of 100% to the normalized mRNA levels obtained for Wt BMDM 3 h after the LPS stimulation.

RNase OUT [Invitrogen], 1 mM phenylmethylsulfonyl fluoride, plus 1 tablet/50 ml of Roche [Indianapolis, IN] inhibitors) and centrifuged at 1400 rpm for 15 min at 4°C. One milligram of supernatant protein was added to 50 μl of 7-methyl GTP (m^7GTP) Sepharose (Amersham), previously precleared with washing buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 1.5 mM $MgCl_2$). Samples were tumbled at 4°C overnight and, after five washes in 1 ml of washing buffer plus 1 mM DTT, were boiled in Laemmli sample buffer and separated on 7–15% SDS–PAGE gels. Western blot analysis was performed to determine coprecipitated 4E-BP1 and eIF4E proteins.

BMDM nucleofection

BMDM were nucleofected with the biscitronic pcDNA3rLuc-polI-RESfLuc plasmid (1 μg/1 × 10^6 cells), generously provided by Alexey Benyumov (University of Minnesota) using the Ingenio Electroporation Kit (Mirus Bio, Madison, WI) and the Nucleofector System (Amaxa Biosystems, Lonza, Cologne, Germany) and performed according to the manufacturer's instructions. Four hours after transfection, cells were washed with RPMI plus 0.5% fetal bovine serum (FBS) and incubated overnight in RPMI, 0.5% FBS, and gentamicin (80 μg/ml). Subsequently, cells were stimulated with LPS (300 ng/ml) for 3 h, and the *Renilla* and Firefly luciferase activities were measured in the cell extracts according to the manufacturer's instructions (Promega, Madison, WI).

Isolation of polyribosomal RNA and Northern blot analysis

BMDMs (5 × 10^6 cells) stimulated or not for 3 h with LPS (300 ng/ml) were washed twice on ice with hypotonic buffer (5 mM Tris-HCl, pH 7.5, 1.5 mM KCl, 2.5 mM $MgCl_2$), lysed in lysis buffer (hypotonic buffer plus 0.5% Triton X-100, 0.5% Na deoxycholate, 120 U/ml of RNase inhibitor, and 3 mM DTT), and centrifuged at 6000 rpm for 8 min at 4°C. After measurement of protein concentration, heparin (1 mg/ml) was added to the extracts. Extract were loaded on a linear sucrose gradient (0.5 and 1.5 M sucrose in 20 mM Tris-HCl, pH 8, 80 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT) and centrifuged in a Beckman SW41 rotor at 36,000 rpm for 2 h at 4°C. Gradients were fractionated, following the RNA absorbance profile at 254 nm, in different fractions. Once collected, fractions were incubated with proteinase K (5 μg/ml) in proteinase K buffer (100 mM Tris-HCl, pH 7.5, 50 mM EDTA, 5% SDS) for 30 min at 37°C. RNA for Northern blot analysis was isolated using phenol/chloroform, and hybridization of Northern blots with the eEF1α and β-actin probes was performed as described previously (Shima *et al.*, 1998).

qRT-PCR

Total RNA was isolated from BMDMs (5 × 10^6 cells) stimulated or not for 3 h with LPS (300 ng/ml), using the miRNeasy Mini Kit and according to the manufacturer's instructions (Qiagen, Valencia, CA). Recruited mRNA to polysomes was isolated using phenol/chloroform and further purified as described previously (del Prete *et al.*, 2007). Briefly, RNA was subjected to a precipitation with 2 M LiCl at –20°C overnight. After centrifugation (12,000 × *g*, 15 min at 4°C), pellets were washed twice with 70% ETOH (prestored at –20°C), air-dried, and resuspended in RNase-free water. qRT-PCR was performed as described previously (Rodriguez *et al.*, 2008). The specific TAQMAN primers (Applied Biosystems, Foster City, CA) TNFα, IL-6, KC, β-actin, and 18Sr were used.

Statistical analysis

Experiments shown were performed at least three times. The graphical data presented, as the means ± SD, were analyzed with Student's *t* test. Values were taken to be statistically significant at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

ACKNOWLEDGMENTS

This work was supported by SAF 2011-24481 and Mutua Madrileña grants to S.A. and by FIS 2009-80145 and Universidad Autónoma de Madrid CM-CCG10-4911 grants to G.S. PD 0325901 was a gift from Philip Cohen, rapamycin was from Victor Calvo, and the bis-citronic pcDNA3rLuc-polIRESfLuc plasmid was generously provided by Alexey Benyumov. We thank Sara Kozma and Amelia Nieto for critical reading of the manuscript and Raquel Cantalejo for technical work. M.L.P. is a recipient of an FPU Fellowship from the Universidad Autónoma de Madrid. S.G. holds a research contract from the Ramón y Cajal Program of Spain.

REFERENCES

Akira S, Takeda K (2004). Toll-like receptor signalling. Nat Rev Immunol 4, 499–511.

Anderson P (2008). Post-transcriptional control of cytokine production. Nat Immunol 9, 353–359.

Anderson P (2010). Post-transcriptional regulons coordinate the initiation and resolution of inflammation. Nat Rev Immunol 10, 24–35.

Anjum R, Blenis J (2008). The RSK family of kinases: emerging roles in cellular signalling. Nat Rev Mol Cell Biol 9, 747–758.

Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P (2007). The selectivity of protein kinase inhibitors: a further update. Biochem J 408, 297–315.

Ballester A, Velasco A, Tobena R, Alemany S (1998). Cot kinase activates tumor necrosis factor-alpha gene expression in a cyclosporin A-resistant manner. J Biol Chem 273, 14099–14106.

Banerjee A, Gugasyan R, McMahon M, Gerondakis S (2006). Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. Proc Natl Acad Sci USA 103, 3274–3279.

Buxade M, Parra JL, Rousseau S, Shpiro N, Marquez R, Morrice N, Bain J, Espel E, Proud CG (2005). The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. Immunity 23, 177–189.

Caivano M, Rodriguez C, Cohen P, Alemany S (2003). 15-Deoxy-delta12,14-prostaglandin J2 regulates endogenous Cot MAPK kinase kinase 1 activity induced by lipopolysaccharide. J Biol Chem 278, 52124–52130.

Carriere A, Cargnello M, Julien LA, Gao H, Bonneil E, Thibault P, Roux PP (2008). Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr Biol 18, 1269–1277.

Cohen P (2009). Targeting protein kinases for the development of anti-inflammatory drugs. Curr Opin Cell Biol 21, 317–324.

Colina R *et al.* (2008). Translational control of the innate immune response through IRF-7. Nature 452, 323–328.

Chaurasia B, Mauer J, Koch L, Goldau J, Kock AS, Bruning JC (2010). Phosphoinositide-dependent kinase 1 provides negative feedback inhibition to Toll-like receptor-mediated NF-kappaB activation in macrophages. Mol Cell Biol 30, 4354–4366.

Chen CY, Shyu AB (1995). AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci 20, 465–470.

Cho J, Tsichlis PN (2005). Phosphorylation at Thr-290 regulates Tpl2 binding to NF-kappaB1/p105 and Tpl2 activation and degradation by lipopolysaccharide. Proc Natl Acad Sci USA 102, 2350–2355.

Das S, Cho J, Lambertz I, Kelliher MA, Eliopoulos AG, Du K, Tsichlis PN (2005). Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. J Biol Chem 280, 23748–23757.

del Prete MJ, Vernal R, Dolznig H, Mullner EW, Garcia-Sanz JA (2007). Isolation of polysome-bound mRNA from solid tissues amenable for RT-PCR and profiling experiments. RNA 13, 414–421.

Dumitru CD *et al.* (2000). TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103, 1071–1083.

Fukao T, Koyasu S (2003). PI3K and negative regulation of TLR signaling. Trends Immunol 24, 358–363.

Gaestel M, Kotlyarov A, Kracht M (2009). Targeting innate immunity protein kinase signalling in inflammation. Nat Rev Drug Discov 8, 480–499.

Gandara ML, Lopez P, Hernando R, Castano JG, Alemany S (2003). The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. Mol Cell Biol 23, 7377–7390.

Gantke T, Sriskantharajah S, Ley SC (2011). Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase. Cell Res 21, 131–145.

Gantner BN, Simmons RM, Canavera SJ, Akira S, Underhill DM (2003). Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med 197, 1107–1117.

Garcia-Martinez JM, Alessi DR (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J 416, 375–385.

Gingras AC, Raught B, Sonenberg N (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68, 913–963.

Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM (2006). Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell 11, 859–871.

Guo H, Ingolia NT, Weissman JS, Bartel DP (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466, 835–840.

Hao S, Baltimore D (2009). The stability of mRNA influences the temporal order of the induction of genes encoding inflammatory molecules. Nat Immunol 10, 281–288.

Hatziapostolou M, Koukos G, Polytarchou C, Kottakis F, Serebrennikova O, Kuliopulos A, Tsichlis PN (2011). Tumor progression locus 2 mediates signal-induced increases in cytoplasmic calcium and cell migration. Sci Signal 4, ra55.

Hellen CU, Sarnow P (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev 15, 1593–1612.

Huo Y, Iadevaia V, Yao Z, Kelly I, Cosulich S, Guichard S, Foster LJ, Proud CG (2012). Stable isotope-labelling analysis of the impact of inhibition of the mammalian target of rapamycin on protein synthesis. Biochem J 444, 141–151.

Jefferies HB, Fumagalli S, Dennis PB, Reinhard C, Pearson RB, Thomas G (1997). Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. EMBO J 16, 3693–3704.

Kaiser F *et al.* (2009). TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. J Exp Med 206, 1863–1871.

Kawai T, Akira S (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637–650.

Kotenko SV, Krause CD, Izotova LS, Pollack BP, Wu W, Pestka S (1997). Identification and functional characterization of a second chain of the interleukin-10 receptor complex. EMBO J 16, 5894–5903.

Laplante M, Sabatini DM (2009). mTOR signaling at a glance. J Cell Sci 122, 3589–3594.

Lopez-Pelaez M, Soria-Castro I, Bosca L, Fernandez M, Alemany S (2011). Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: implications for NO synthase 2 expression. Eur J Immunol 41, 1733–1741.

Ma L, Chen Z, Erdjument-Bromage H, Tempst P, Pandolfi PP (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 121, 179–193.

Marzec M, Liu X, Wysocka M, Rook AH, Odum N, Wasik MA (2011). Simultaneous inhibition of mTOR-containing complex 1 (mTORC1) and MNK induces apoptosis of cutaneous T-cell lymphoma (CTCL) cells. PLoS One 6, e24849.

Mazumder B, Li X, Barik S (2010). Translation control: a multifaceted regulator of inflammatory response. J Immunol 184, 3311–3319.

Meyuhas O (2000). Synthesis of the translational apparatus is regulated at the translational level. Eur J Biochem 267, 6321–6330.

Mielke LA, Elkins KL, Wei L, Starr R, Tsichlis PN, O'Shea JJ, Watford WT (2009). Tumor progression locus 2 (Map3k8) is critical for host defense against *Listeria monocytogenes* and IL-1 beta production. J Immunol 183, 7984–7993.

O'Neill LA, Bowie AG (2007). The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7, 353–364.

Patursky-Polischuk I, Stolovich-Rain M, Hausner-Hanochi M, Kasir J, Cybulski N, Avruch J, Ruegg MA, Hall MN, Meyuhas O (2009). The TSC-mTOR pathway mediates translational activation of TOP mRNAs by insulin largely in a raptor- or rictor-independent manner. Mol Cell Biol 29, 640–649.

Pearce LR, Komander D, Alessi DR (2010). The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol 11, 9–22.

Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, Agol VI, Hellen CU (2001). Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci USA 98, 7029–7036.

Rodriguez C, Lopez P, Pozo M, Duce AM, Lopez-Pelaez M, Fernandez M, Alemany S (2008). COX2 expression and Erk1/Erk2 activity mediate Cot-induced cell migration. Cell Signal 20, 1625–1631.
Rodriguez C, Pozo M, Nieto E, Fernandez M, Alemany S (2006). TRAF6 and Src kinase activity regulates Cot activation by IL-1. Cell Signal 18, 1376–1385.
Rousseau S, Morrice N, Peggie M, Campbell DG, Gaestel M, Cohen P (2002). Inhibition of SAPK2a/p38 prevents hnRNP A0 phosphorylation by MAPKAP-K2 and its interaction with cytokine mRNAs. EMBO J 21, 6505–6514.
Rousseau S, Papoutsopoulou M, Symons A, Cook D, Lucocq JM, Prescott AR, O'Garra A, Ley SC, Cohen P (2008). TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. J Cell Sci 121, 149–154.
Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J (2004). Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci USA 101, 13489–13494.
Roux PP, Blenis J (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68, 320–344.
Roux PP, Shahbazian D, Vu H, Holz MK, Cohen MS, Taunton J, Sonenberg N, Blenis J (2007). RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. J Biol Chem 282, 14056–14064.
Sachs AB, Varani G (2000). Eukaryotic translation initiation: there are (at least) two sides to every story. Nat Struct Biol 7, 356–361.
Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307, 1098–1101.
Shima H, Pende M, Chen Y, Fumagalli S, Thomas G, Kozma SC (1998). Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J 17, 6649–6659.
Soria-Castro I, Krzyzanowska A, Pelaez ML, Regadera J, Ferrer G, Montoliu L, Rodriguez-Ramos R, Fernandez M, Alemany S (2010). Cot/tpl2 (MAP3K8) mediates myeloperoxidase activity and hypernociception following peripheral inflammation. J Biol Chem 285, 33805–33815.
Stafford MJ, Morrice NA, Peggie MW, Cohen P (2006). Interleukin-1 stimulated activation of the COT catalytic subunit through the phosphorylation of Thr290 and Ser62. FEBS Lett 580, 4010–4014.
Tamura T, Yanai H, Savitsky D, Taniguchi T (2008). The IRF family transcription factors in immunity and oncogenesis. Annu Rev Immunol 26, 535–584.
Tang H, Hornstein E, Stolovich M, Levy G, Livingstone M, Templeton D, Avruch J, Meyuhas O (2001). Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol Cell Biol 21, 8671–8683.
Thomson AW, Turnquist HR, Raimondi G (2009). Immunoregulatory functions of mTOR inhibition. Nat Rev Immunol 9, 324–337.
Thoreen CC, Chantranupong L, Keys HR, Wang T, Gray NS, Sabatini DM (2012). A unifying model for mTORC1-mediated regulation of mRNA translation. Nature 485, 109–113.
Topisirovic I, Svitkin YV, Sonenberg N, Shatkin AJ (2011). Cap and cap-binding proteins in the control of gene expression. Wiley Interdiscip Rev RNA 2, 277–298.
Vougioukalaki M, Kanellis DC, Gkouskou K, Eliopoulos AG (2011). Tpl2 kinase signal transduction in inflammation and cancer. Cancer Lett 304, 80–89.
Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG (2001). Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J 20, 4370–4379.
Waterfield MR, Zhang M, Norman LP, Sun SC (2003). NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. Mol Cell 11, 685–694.
Weichhart T, Saemann MD (2009). The multiple facets of mTOR in immunity. Trends Immunol 30, 218–226.

Supplementary Table 1

Quantification of Erk1/2, Akt, SGK1, and S6K1 phosphorylation in LPS-stimulated Wt and Cot/tpl2 KO bone marrow derived macrophages.

	Wt						Cot/tpl2 KO						
	0	0.5	1	1.5	2	4	0	0.5	1	1.5	2	4	LPS (h)
P-Erk1/2	1	3.1	2.2	1.5	1.3	1.1	0.7	0.7	0.7	0.7	0.7	0.6	
P-T308 Akt	1	2.6	1.7	2.0	2.0	1.8	1.2	2.6	1.9	1.9	1.9	1.6	
P-S473 Akt	1	3.8	3.2	1.8	0.8	0.9	0.7	1.3	2.1	1.7	1.2	0.8	
P-S422 SGK1	1	1.3	2.1	1.8	0.7	0.6	0.6	0.7	1.2	0.8	0.5	0.4	
P-T389 S6K1	1	5.3	5.8	3.3	2.9	1.7	0.9	1.7	3.1	1.2	1.1	0.9	

Wt and Cot/tpl2 KO bone marrow derived macrophages were stimulated with LPS (300 ng/ml) and after the indicated times P-Erk1/2, P-T308 Akt, P-S473 Akt, P-S422 SGK1, and P-T389 S6K1 levels were measured by Western-blot. Total Erk1/2, Akt, SGK1, and S6K1 levels were determined as a protein loading control. Data are the means of at least 4 independent experiments of P-Erk1/2, P-T308 Akt, P-S473 Akt, P-S422 SGK1, and P-T389 S6K1 fold induction relative to the Wt 0 time point, after normalizing values to respectively total Erk1/2, Akt, SGK1, and S6K1.

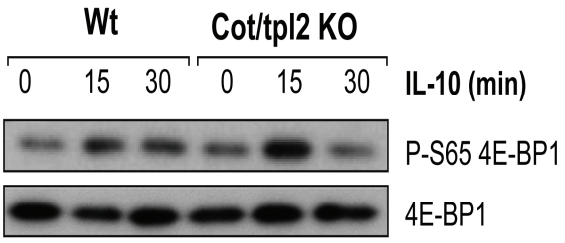


Figure S1. IL-10-induced P-S65 4E-BP1 phosphorylation in BMDM is independent of Cot/tpl2 expression. Wt and Cot/tpl2 KO BMDM were stimulated with IL-10 (50 ng/ml) for 30 and 60 min and the expression levels of P-S65 4E-BP1 and 4E-BP were determined in Western-blots. One representative experiment of the 3 performed is shown.

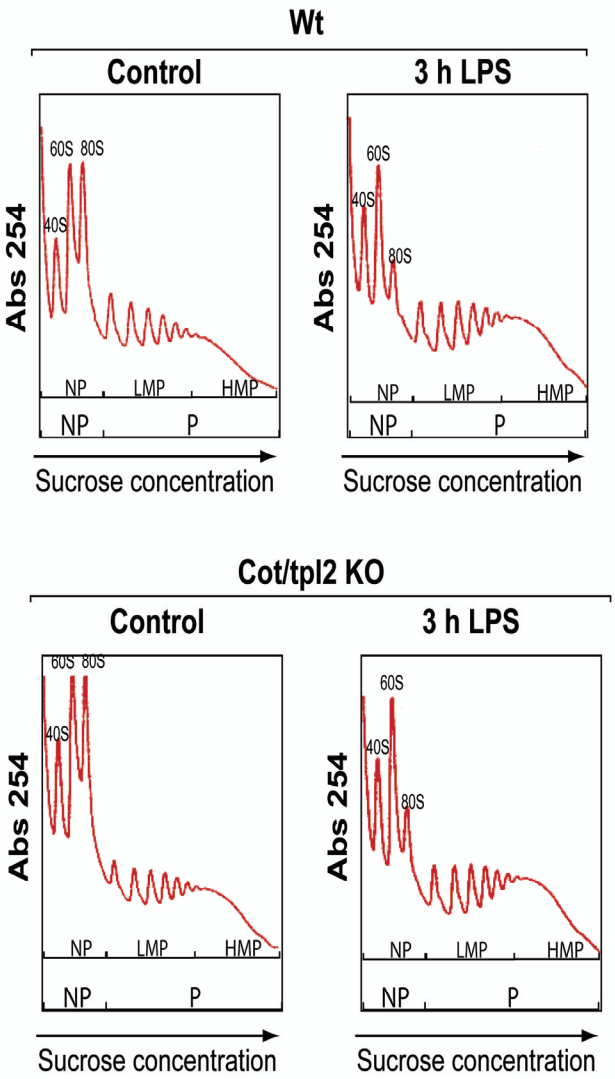


Figure S2. Representative ribosomal profiling of cell lysates of Wt and Cot/tpl2 KO BMDM. Cell lysates of Wt and Cot/tpl2 KO BMDM stimulated for 3 h or not with LPS (300 ng/ml) were subjected to sucrose gradient and fractionated in different mRNA fractions. **NP**, non translated mRNA (non polysomal components, consisting of non polysomal mRNAs and non translating free 40S and 60S ribosomal); **P**, translated mRNA (polysomes): **LMP**, moderately translated mRNA (low molecular weight polysomes); and **HMP**, actively translated mRNA (high molecular weight polysomes fraction).

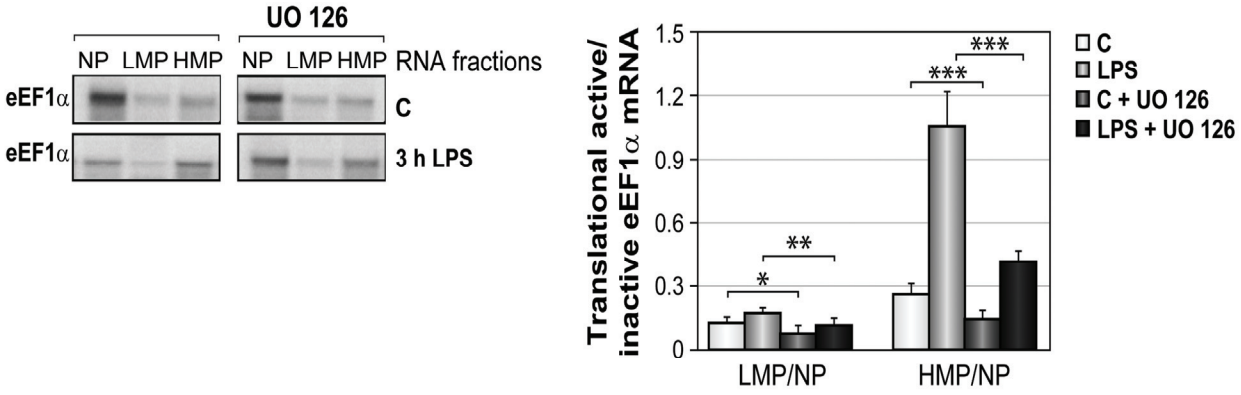


Figure S3. UO 126 down-regulates eEF1α polysomal recruitment in LPS-stimulated BMDM. Cell lysates of Wt BMDM preincubated or not for 30 min with the MKK1/2 inhibitor UO 126 (10 μM) and subsequently stimulated for 3 h or not with LPS (300 ng/ml) were subjected to sucrose gradient and 3 mRNA fractions were pooled: non translated mRNA (non polysomal components, consisting of non polysomal mRNAs and non translating free 40S and 60S ribosomal, NP), moderately translated mRNA (low molecular weight polysomes, LMP), and actively translated mRNA (high molecular weight polysomes fraction, HMP). Northern-blot analysis showing eEF1α mRNA expression in the NP, LMP, and HMP fractions of non-stimulated and 3 h LPS-stimulated Wt and Cot/tpl2 KO BMDM was performed. On the left one representative experiment is shown and on the right, graph represents the means + SD from 3 independent experiments of the quantification of the LMP/NP and HMP/NP eEF1α mRNA ratios in the different cell conditions

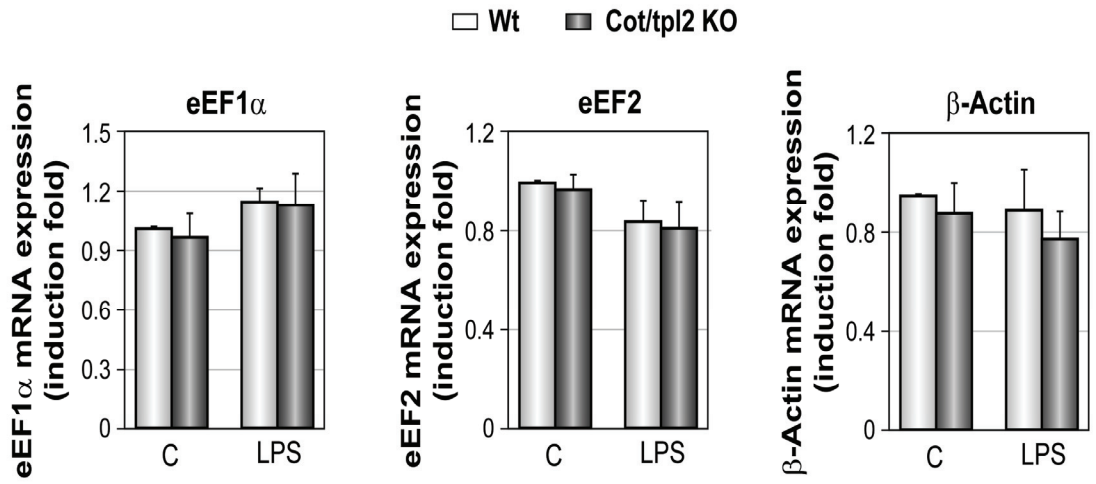


Figure S4. eEF1α, eEF2, and β-Actin mRNA levels in Wt and Cot/tpl2 KO BMDM following LPS stimulation. Total mRNA levels were determined by qRT-PCR in Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h with LPS (300 ng/ml). eEF1α, eEF2, and 18Sr mRNAs were determined by Sybr green qRT-PCR using the following primers: eEF1α, sense 5′- ACACGTAGATTCCGGCAAGT-3′ and antisense′ 5 -AGGAGCCCTTTCCCATCTC-3; eEF2, sense 5′- GTTGACGTCAGCGGTCTCTT-3′ and antisense 5′- GCACGGATCTGATCTACTGTGA-3′, and their expression levels were normalized in each assay to levels of 18Sr mRNA, using the primers, sense 5′ CCA GTA AGT GCG GGT CAT AAG C and antisense 5′ CCT CAC TAA ACC ATC CAA TCG G. The β-Actin primers were from TAQMAN (Applied Biosystems) and were normalized to levels of 18Sr mRNA expression with TAQMAN primers in each assay. Graphs represent the means + SD (n=5), given the value of 1 to the obtained one in non stimulated Wt BMDM.

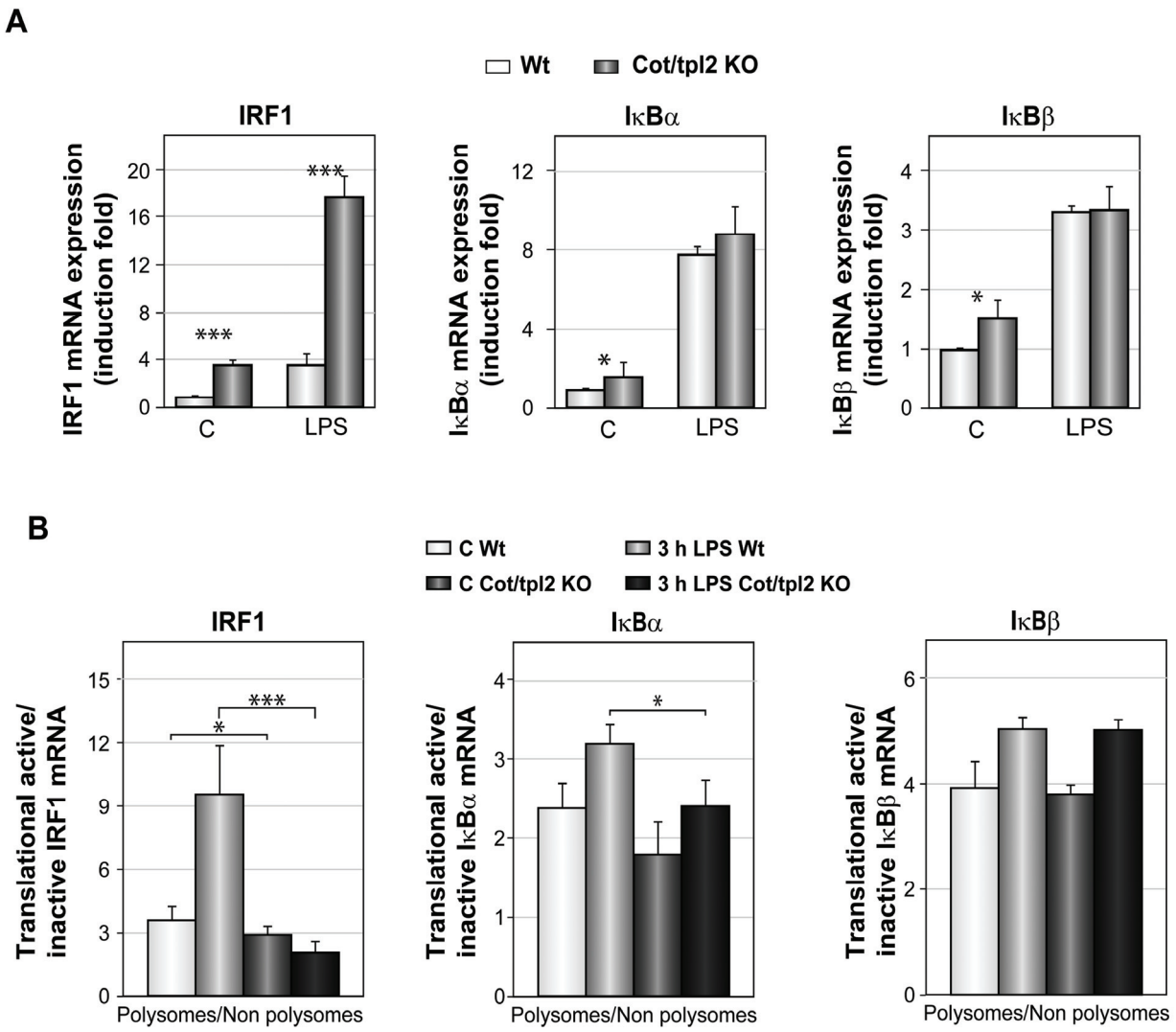


Figure S5. Polysomal recruitment of IRF1, IκBα, and IκBβ mRNA in LPS-stimulated Wt and Cot/tpl2 KO BMDM. A) Total IRF1, IκBα, and IκBβ mRNA levels were determined in Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h with LPS (300 ng/ml) by qRT-PCR analysis. Expression levels of the different transcripts were normalized to levels of 18Sr mRNA expression in each assay. Graphs show the mean + SD from 5 independent experiments, given the value of 1 to the one obtained in control Wt BMDM. **B)** IRF1, IκBα, and IκBβ mRNA expression within polysomal and non polysomal RNA fractions described in Figure 5, was analyzed by qRT-PCR. Graphs represent the means + SD from 5 independent experiments of the quantification of the Polysomes/Non polysomes mRNA ratio in the different cell conditions.

Capítulo II

Sterile inflammation in Acetaminophen-induced liver injury is mediated by Cot/tpl2

Sanz-García, C.; Ferrer-Mayorga, G.; González-Rodríguez, A.; Valverde, A.M.; Martín-Duce, A.; Velasco-Martín, J.P.; Regadera, J.; Fernandez, M.; Alemany, S.

The Journal of Biological Chemistry (288) 15342-15351 (2013)

Dosis tóxicas de APAP inducen una necrosis inicial en el hígado que supone el principio de un proceso de inflamación estéril que agrava el desarrollo de la necrosis hepática. En este trabajo hemos estudiado el papel de Cot/tpl-2 en toxicidad hepática inducida por sobredosis de APAP. Tras la inyección i.p. de APAP, ratones deficientes en Cot/tpl-2 presentan un menor grado de inflamación, así como niveles disminuidos de citoquinas pro-inflamatorias en el hígado resultando en una menor necrosis hepática. Por otra parte, la estimulación de macrófagos con DAMPs, generados tras la necrosis celular, activa distintas vías de señalización intracelular entre las que se encuentra el eje Cot/tpl-2--MKK1/2--Erk1/2. Estos datos indican por primera vez que Cot/tpl-2 participa en la generación de inflamación estéril.

Estudios de supervivencia con ratones Wt y Cot/tpl-2 KO realizados tras la inyección i.p. de APAP, revelan que la deficiencia de Cot/tpl-2 previene parcialmente la toxicidad generada por una sobredosis del fármaco. Así, los niveles en sangre de las transaminasas ALT y AST, marcadores de daño hepático, están incrementados 24 horas después de la inyección i.p. de APAP, siendo dicho aumento mucho mayor en ratones Wt que en ratones Cot/tpl-2 KO. Los niveles de LDH, marcador de necrosis celular, también están incrementados en sangre de los ratones Wt respecto a los Cot/tpl-2 KO. La fosforilación de JNK, otro marcador de daño hepático, también está reducida por la deficiencia de Cot/tpl-2. Además tinciones con hematoxilina y eosina de hígado de diferentes ratones tratados con APAP, muestran una congestión hepática 3 veces superior en ratones Wt que en ratones Cot/tpl-2 KO. Por otro lado ratones que expresan la proteína Cot/tpl-2 inactiva también presentan, una mayor resistencia a la toxicidad por APAP que los ratones Wt, indicando que es la actividad Cot/tpl-2 la que participa en el proceso de toxicidad producido por sobredosis APAP.

Como consecuencia de un daño hepático inicial tras la inyección i.p. de APAP se produce un reclutamiento de leucocitos al hígado. El análisis de los leucocitos infiltrados en hígado tras 18 horas de la inyección de APAP, muestra que Cot/tpl-2 participa en dicho reclutamiento. Un análisis más detallado de esta población confirma que neutrófilos y macrófagos son las dos poblaciones reclutadas en mayor medida en ratones Wt, estando su reclutamiento atenuado en el hígado de ratones Cot/tpl-2 KO. Además, Cot/tpl-2 también participa en el reclutamiento de las células NKT ($CD3^+NK1.1^+$) al hígado. Por otra parte, ratones Cot/tpl-2 KO muestran menores niveles de mRNA de IL-1α, IL-1β, IL-6 y TNFα, y una subida de los niveles de IL-10 en el hígado de los ratones Wt tras la administración de APAP, estos datos que concuerdan con la cuantificación de las diferentes citoquinas en suero.

Para determinar sí Cot/tpl-2 modula la toxicidad inducida por APAP directamente en hepatocitos, hepatocitos inmortalizados WT y Cot/tpl-2 KO incubados con distintas concentraciones de APAP en presencia o ausencia de TNFα, citoquina que incrementa la toxicidad del APAP, mostraron niveles de supervivencia muy parecidos; obteniéndose también niveles muy similares de LDH en el medio extracelular y de P-JNK intracelulares en hepatocitos Wt y Cot/tpl-2 KO. Todos estos datos en conjunto indican que Cot/tpl-2 modula "*in vivo*" la toxicidad inducida por APAP pero no en hepatocitos aislados.

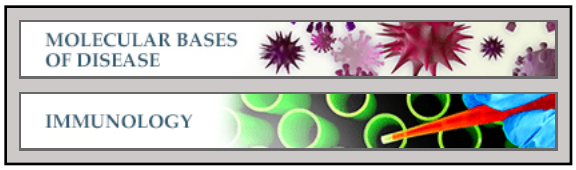
La toxicidad inducida por APAP produce una necrosis inicial en el hígado, liberando moléculas que actúan como DAMPs y que señalizan en otras células. La estimulación de macrófagos con DAMPs generados a partir de hepatocitos, incrementa la concentración de IL-10, IL-1α, IL-1β, IL-6 y TNFα en el sobrenadante extracelular, estando la producción de estas citoquinas mediadas por Cot/tpl-2. Además un análisis del estado de activación de las distintas MAPKs en dichos macrófagos revela que la deficiencia en Cot/tpl-2 reduce la activación de Erk1/2 y JNK, sin alterar la fosforilación de p38α. Tras la estimulación con DAMPs, Erk1/2 tiene dos picos de activación, uno a 7 min y otro más tardío a 30 min. Así, solo el segundo pico de activación de Erk1/2 está mediado por Cot/tpl-2. Cot/tpl-2 regula también la activación de JNK tanto en macrófagos derivados de medula ósea como en macrófagos peritoneales estimulados por DAMPs.

Posteriormente evaluamos el papel de Cot/tpl-2 en la inflamación estéril "*in vivo*" generada por los DAMPs, inyectándolos i.p. en ratones Wt y Cot/tpl-2 KO. Tras 18 horas, se analizaron las células presentes en el peritoneo mediante citometría de flujo. La deficiencia de Cot/tpl-2 conduce a una reducción en el reclutamiento de neutrófilos y macrófagos a la cavidad peritoneal. Análisis del estado oxidativo celular de las células reclutadas al peritoneo, muestra una pequeña disminución en leucocitos Cot/tpl-2 KO respecto a leucocitos Wt en estado basal, dicha diferencia se incrementa tras la estimulación con PMA. Finalmente, la medida de los niveles de IL-10, IL-1α, IL-1β e IL-6 en la cavidad peritoneal de estos ratones indica que Cot/tpl-2 KO también regula la producción de las mismas en ambas condiciones. Estos datos ponen de manifiesto el papel necesario de Cot/tpl-2 en la inflamación estéril "in vivo".

El doctorando llevó a cabo el mantenimiento de las colonias de ratones utilizados, así como los tratamientos necesarios para la parte experimental. Se encargó de todos los ensayos realizados con macrófagos derivados de médula ósea y los peritoneales, así como de los ensayos "in vivo" realizados con DAMPs. Además, ayudó en la redacción del manuscrito y en la creación y corrección de las diferentes figuras.

Molecular Bases of Disease:

Sterile Inflammation in Acetaminophen-induced Liver Injury Is Mediated by Cot/tpl2



Carlos Sanz-Garcia, Gemma Ferrer-Mayorga, Águeda González-Rodríguez, Ángela M. Valverde, Antonio Martín-Duce, Juan P. Velasco-Martín, Javier Regadera, Margarita Fernández and Susana Alemany

J. Biol. Chem. 2013, 288:15342-15351.
doi: 10.1074/jbc.M112.439547 originally published online April 9, 2013

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 21, pp. 15342–15351, May 24, 2013

Sterile Inflammation in Acetaminophen-induced Liver Injury Is Mediated by Cot/tpl2*[S]

Received for publication, November 29, 2012, and in revised form, April 3, 2013 Published, JBC Papers in Press, April 9, 2013, DOI 10.1074/jbc.M112.439547

Carlos Sanz-Garcia[‡1], Gemma Ferrer-Mayorga[‡], Águeda González-Rodríguez[‡§2], Ángela M. Valverde[‡§], Antonio Martín-Duce[‡], Juan P. Velasco-Martín[¶], Javier Regadera[¶], Margarita Fernández[‡], and Susana Alemany[‡3]

From the [‡]Instituto Investigaciones Biomédicas Alberto Sols, CISC-UAM. 28029 Madrid, Spain, [§]Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas, CIBERDEM, ISCIII, Spain, and the [¶]Dpto. de Anatomía, Histología y Neurociencia Fac. Medicina, UAM 28029 Madrid, Spain

Background: MAP3K8 (Cot/tpl2) activates MKK1/2-Erk1/2 upon stimulation of receptors from the Toll-like/interleukin-1 receptor superfamily.
Results: Cot/tpl2 plays an essential role in acetaminophen-induced liver injury by modulating the generation of inflammatory signals induced by necrotic cells.
Conclusion: Sterile inflammatory processes triggered by tissue damage are modulated by Cot/tpl2.
Significance: Cot/tpl2 contributes to the development of pathologies associated with inflammation triggered by damage-associated molecular patterns.

Cot/tpl2 (MAP3K8) activates MKK1/2-Erk1/2 following stimulation of the Toll-like/IL-1 receptor superfamily. Here, we investigated the role of Cot/tpl2 in sterile inflammation and drug-induced liver toxicity. Cot/tpl2 KO mice exhibited reduced hepatic injury after acetaminophen challenge, as evidenced by decreased serum levels of both alanine and aspartate aminotransferases, decreased hepatic necrosis, and increased survival relative to Wt mice. Serum levels of both alanine and aspartate aminotransferases were also lower after intraperitoneal injection of acetaminophen in mice expressing an inactive form of Cot/tpl2 compared with Wt mice, suggesting that Cot/tpl2 activity contributes to acetaminophen-induced liver injury. Furthermore, Cot/tpl2 deficiency reduced neutrophil and macrophage infiltration in the liver of mice treated with acetaminophen, as well as their hepatic and systemic levels of IL-1α. Intraperitoneal injection of damage-associated molecular patterns from necrotic hepatocytes also impaired the recruitment of leukocytes and decreased the levels of several cytokines in the peritoneal cavity in Cot/tpl2 KO mice compared with Wt counterparts. Moreover, similar activation profiles of intracellular pathways were observed in Wt macrophages stimulated with Wt or Cot/tpl2 KO damage-associated molecular patterns. However, upon stimulation with damage-associated molecular patterns, the activation of Erk1/2 and JNK was deficient in Cot/tpl2 KO macrophages compared with their Wt counterparts; an effect accompanied by weaker release of several cytokines, including IL-1α, an important component in the development of sterile inflammation. Taken together, these findings indicate that Cot/tpl2 contributes to acetaminophen-induced liver injury, providing some insight into the underlying molecular mechanisms.

The liver is a crucial metabolic organ and is highly susceptible to drug toxicity. Acetaminophen (APAP)[4] is one of the best-selling analgesics and antipyretics on the market in the United States and Western Europe, although it also accounts for ~50% of all cases of acute liver failure (1–3). APAP overdose generates *N*-acetyl-*p*-benzoquinone imine, which depletes hepatic glutathione and promotes oxidative stress, leading finally to hepatocyte necrosis (reviewed in Refs. 4, 5). Necrosis occurs when cells die rapidly in response to acute injury, ultimately provoking the release of intracellular constituents into the surrounding milieu (6). Some of these components released belong to a group of molecules known as damage-associated molecular patterns (DAMPs). Outside of their physiological environment, these molecules behave as "danger sensors" and are capable of triggering an inflammatory response in a similar way as previously described for the pathogen-associated molecular patterns (PAMPs) (reviewed in Refs. 7, 8). Similar to pathogen-induced inflammation, sterile inflammation initiated by necrotic cells alerts the host tissue to host damage by activating the innate immune system (9, 10). Initially, this process is manifested by the production of pro-inflammatory mediators and triggering the recruitment of leukocytes to the damaged tissue (7, 11–13). However, when excessive, this inflammatory response can contribute to severe organ damage and dysfunction (7, 11, 14). DAMPs include a wide variety of molecules, and the number of known DAMPs is growing continually (7, 15–18).

* This work was supported by Grants SAF 2011-24481 (to S. A.) and SAF 2012-33283 (to A. M. V.) from MINECO (Spain) Comunidad de Madrid S2010/BMD-2423, EFSD/Amylin Programme 2011 (to A. M. V.) and a grant from the Mutua Madrileña (to S. A.).
[S] This article contains supplemental Figs. S1–S9.
[1] Recipient of an FPI fellowship.
[2] Recipient of a CIBERDEM (ISCIII) postdoctoral contract.
[3] To whom correspondence should be addressed: CSIC-UAM Arturo Duperier 4, 28029 Madrid, Spain. Tel.: 34914975418; Fax: 34915854401; E-mail: salemany@iib.uam.es.

[4] The abbreviations used are: APAP, acetaminophen; BMDM, bone marrow-derived macrophages; DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; LPS, lipopolysaccharide; i.p., intraperitoneal; LDH, lactate dehydrogenase; MFI, mean fluorescence intensity.

DAMPs activate a variety of receptor types in different cells, among which are the members of the pattern-recognition receptor (PRR) family, including the receptors of the Toll-like receptor (TLR) family (7, 10, 16, 18, 19). Upon activation, all TLRs (except TLR3) recruit the MyD88 adaptor protein, which mediates activation of the p38α and JNK MAP pathways, and the canonical IKKs, IKKα, and IKKβ (20, 21). Activated IKKβ phosphorylates p105 NFκB, marking it for partial proteolysis. In resting cells, Cot/tpl2 (MAP3K8) forms an inactive complex with p105 NFκB and ABIN2, from which Cot/tpl2 is released following proteolysis of p105 NFκB (reviewed in Refs. 22, 23). The dissociated and activated Cot/tpl2 stimulates MKK1/2 and consequently Erk1/2 (24–26), and it is subsequently rapidly degraded through the proteasome pathway (27, 28). Cot/tpl2 is the only MAP3K to activate the Erk1/2 pathway in response to both TLR activation and IL-1 or TNFα stimulation (29, 30) in different cell types, including macrophages, epithelial, and stellate cells (25, 29, 31). Moreover, Cot/tpl2 can also activate the MAP kinases JNK and p38α in certain conditions, in a cell type- and stimulus-specific manner (22, 30). Thus, Cot/tpl2 fulfills a role in TLR, IL-1, and TNFα intracellular signaling that cannot be substituted by any other protein (22). Accordingly, Cot/tpl2 represents an interesting anti-inflammatory target, given that it does not modulate Erk1/2 phosphorylation via the activation of one or more Raf isoforms (32, 33). Cot/tpl2 contributes to generation of inflammatory nociception and neutrophil recruitment in mice hindpaw upon zymosan injection (34), promotes the Crohn-like inflammatory bowel disease (35, 36), and plays a role in the development of acute pancreatitis (37). However, the double APC KO Cot/tpl2 KO mice show increased deficiency intestinal inflammation and tumorigenesis compared with APC KO Cot/tpl2 Wt and Cot/tpl2 deficiency also increases the ovalbumin-induced airway allergic response (38).

Here, we show that Cot/tpl2 mediates, both *in vitro* and *in vivo*, DAMP-induced IL-1α production. Cot/tpl2 participates in the recruitment of leukocytes to damaged tissue in mice injected intraperitoneally with DAMPs or with a toxic dose of APAP. Furthermore, APAP-induced liver injury is markedly reduced in Cot/tpl2-deficient mice. Taken together, these data indicate that Cot/tpl2 contributes to drug-induced toxicity associated with cell necrosis.

EXPERIMENTAL PROCEDURES

Cells and Stimuli—Immortalized Wt and Cot/tpl2 KO hepatocytes were generated from pools of 4–6 livers obtained from Wt or Cot/tpl2 KO neonatal mice (3.5–4-day-old) that were digested with collagenase and then cultured. The cells were subsequently immortalized with the SV40 Large T antigen, as described previously (39). Hepatocytes were incubated for 18 h with APAP (Sigma-Aldrich) and/or TNFα (Peprotech), and their viability was then assayed using the MTT kit (Roche), according to the manufacturer's instructions. DAMPs were generated from Wt and Cot/tpl2 KO hepatocytes as described previously (12, 13) with minor modifications: prior to 1-h heat-shock at 60 °C, the hepatocytes were washed five times with PBS and resuspended in DMEM-HEPES. Bone marrow-derived macrophages (BMDM) or peritoneal macrophages were generated as described previously (26) and then stimulated with DAMPs (300 μg/ml), LPS (300 ng/ml, Sigma-Aldrich), or PMA (10 μM, Sigma-Aldrich). When necessary, the IKKβ inhibitor B1605906 (10 μM), the JNK inhibitor SP 600125 (12 μm), or the Erk1/2 inhibitor PD 0325901 (0.5 μM), gifts from Sir Philip Cohen (Dundee), were added prior to stimulation.

Animals and Animal Treatments—C57BL/6 Wt, C57BL/6 KO, and C57BL/6 Cot/tpl2 KD littermates were generated from the crossing of heterozygous mice (34), and they were used for experiments at 10–12 weeks of age. All animals were handled in accordance with institutional guidelines for the care and use of laboratory animals in research. The mice were fasted overnight before treatment, and the APAP solution administered (10 mg/ml) was prepared freshly in warmed (55 °C) PBS that was cooled to 35 °C before it was injected. For survival studies, 600 g/kg APAP (intraperitoneal) was administered to mice. A comprehensive laboratory animal monitoring system (TSE) was used to analyze metabolic behavior 4 h after injection, measuring O_2 consumption and CO_2 production every 10 min for 48 h. The time of death was determined as the point at which the respiratory exchange ratio (RER; O_2/CO_2) became zero or negative. To evaluate hepatotoxicity the animals were administered intraperitoneally (i.p.) either with PBS (control) or with APAP (450 g/kg), food was withheld for 4 h, and at the times indicated, the mice were sacrificed. Liver tissue was taken from each mouse, immediately ground into small fragments, frozen in liquid nitrogen, and stored at −80 °C. Blood was collected, and the serum was stored at −80 °C until use. The ALT, AST, and lactate dehydrogenase (LDH) levels were determined (at the UCM, Madrid), and cytokine levels were assessed by Luminex analysis (at the CNB, Madrid). Peritoneal leukocyte recruitment in mice was measured 18 h after injection of liver homogenate (32 mg, intraperitoneal) or of DAMPs generated from hepatocytes (1.7 mg, 35×10^6 cells). Peritoneal cells were collected as described previously (12, 13, 34). Hepatic non-parenchymal cells were isolated as described previously (40). To analyze liver damage, tissue samples were rinsed with saline and fixed by immersion in 4% formalin for 24 h and treated with hematoxylin and eosin (H&E) staining as described previously (34). The analysis of the necrotic areas as well as the quantification (Image J) of the liver areas with congestive changes occupied by erythrocytes (including the intravascular and hemorrhagic areas) were performed in seven randomly selected microscopic fields from each sample using a ×10 objective.

Flow Cytometry—Cells ($0.3–0.5 \times 10^6$ cells/test) were incubated with CD16/32 (2.4G2, Cultek) for 20 min at room temperature, and they were subsequently stained for 1 h at 4 °C in the dark with the following antibodies (5 μg/ml): CD3-PECy7 (Hamster IgG, eBioscience), CD11a-PECy7 (rat IgG2ak, Pharmingen), CD11b-(Mac1)-PECy7 (rat IgG2bk anti-mouse, eBioscience), CD45-FITC (rat IgG, Beckman), F4/80-APC (rat IgG2ak, eBioscience), Ly6G-PE (rat IgG2ak, Pharmingen), and NK1.1-APC (mice IgG2ak anti-mouse, Pharmingen), or their corresponding isotype controls (Pharmingen, eBioscience). After three washes, Perfect-Count microspheres (Cytognos) were added to quantify the exact number of cells. Flow cytometry analysis was performed using the CXP program. To determine the oxidative burst of peritoneal leukocytes, 3×10^5 cells were resuspended in PBS, incubated in the dark with 10 μM

2′,7′-dichlorofluorescin diacetate (Invitrogen), and subsequently incubated in the presence or absence of 10 μM PMA for 15 min at 37 °C. After several washes, the cells were analyzed by flow cytometry.

Western Blot and RT-PCR Analysis—Homogenized liver extracts and cell extracts were analyzed in Western blots (41), probed with primary antibodies raised against the following proteins: Cot/tpl2, Erk2, p38α, and p52 JNK2 (Santa Cruz Biotechnology); P-S933 p105 NFκB, P-T202/Y204 Erk1/2, and P-T180/Y182 p38α (Cell Signaling); and P-T183/Y185 p48/p52 JNK (Invitrogen). Secondary antibodies raised against rabbit (Cell Signaling), goat (DAKO), and mouse (Amersham Biosciences) were used to detect the primary antibodies. RNA extraction and RT-PCR analysis were performed as described previously (42). The specific TAQMAN primers (Applied Biosystems) IL-1α, IL-1β, IL-6, IL-10, TNFα, and β-actin were used.

Statistical Analysis—Data are presented as the mean ± S.D., and they were analyzed using the Student's *t* test. Values were considered statistically significant at $p < 0.05$: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$.

RESULTS

Cot/tpl2 Participates in APAP-induced Liver Damage—Following APAP administration (600 g/kg, intraperitoneal), 12% survival was observed in Wt mice in the 15 to 30 h following injection, whereas all the Cot/tpl2 KO mice survived for the duration of the experiment (Fig. 1*A*). The serum levels of ALT, AST, and LDH were all lower in APAP-treated (450 g/kg, intraperitoneal) Cot/tpl2 KO mice than in their Wt counterparts (Fig. 1*B* and supplemental Fig. S1). Increased hepatic P-JNK levels, as a marker of liver damage, were observed 6 h after APAP-induced liver injury (4, 43), and Cot/tpl2 deficiency decreased JNK phosphorylation in APAP-injected mice (supplemental Fig. S1). Moreover, the liver of Cot/tpl2 KO mice revealed a marked histological attenuation of liver injury compared with the severe centrilobular necrosis and hemorrhage observed in Wt animals (Fig. 1*C*), indicating that Cot/tpl2 deficiency protected against APAP-induced hepatotoxicity. In Cot/tpl2 KD mice that express an inactive version of Cot/tpl2 (42), the levels of AST and ALT after APAP treatment were also diminished compared with those in Wt mice (Fig. 1*D*). These data indicate that APAP-induced liver injury is modulated by Cot/tpl2 activity rather than by modifications in the Cot/tpl2-ABIN2-p105 NFκB complex, because of Cot/tpl2 knockdown.

APAP-induced liver injury triggers the recruitment of leukocytes to the liver (8, 44, 45). Upon intraperitoneal injection of PBS, similar numbers of hepatic leukocytes were detected in Wt and Cot/tpl2 KO mice; yet APAP injection produced a 3-fold increase in the number of hepatic leukocytes ($CD45^+$) in Wt mice, whereas only a 1.5-fold increase was observed in APAP-treated Cot/tpl2 KO mice (Fig. 2*A*). Analysis of the leukocytes recruited revealed that Cot/tpl2 deficiency mainly decreased the number of infiltrated neutrophils ($Ly6G^+$) and macrophages ($F4/80^+$: Fig. 2, *B* and *C*). In the neutrophils, neither Cot/tpl2 deficiency nor APAP treatment altered the mean fluorescence intensity (MFI) of the CD11a and CD11b activation markers. However, the macrophages recruited to the liver following APAP administration had a higher CD11b MFI (44), an effect that was less pronounced in Cot/tpl2 KO macrophages (Fig. 2*D*). Following APAP challenge, Cot/tpl2 also triggered the recruitment of NKT ($CD3^+NK1.1^+$) cells to the liver (Fig. 2*B*). Moreover, the serum IL-1α, IL-1β, and IL-6 levels were lower in APAP-treated Cot/tpl2 KO mice than in Wt mice, while Cot/tpl2 deficiency promoted an increase in IL-10 levels (Fig. 3*A*). Similarly, 6 h upon APAP challenge, the liver of Cot/tpl2 KO mice showed decreased mRNA levels of IL-1α, IL-1β, IL-6, and TNFα, but higher IL-10 levels compared with Wt-treated mice (Fig. 3*B*).

To determine whether Cot/tpl2 also modulates APAP-induced hepatotoxicity in isolated hepatocytes, immortalized Wt and Cot/tpl2 KO hepatocytes were incubated with different concentrations of APAP for 18 h. Similar survival rates were observed for both Wt and Cot/tpl2 KO cells in the presence of APAP (supplemental Fig. S2), and although the presence of TNFα along with APAP further increased cell toxicity, Cot/tpl2 did not appear to modulate hepatocyte cell death in either of these conditions (Fig. 4*A*). Accordingly, similar LDH levels, as a consequence of cell necrosis, were detected in the supernatant of both Wt and Cot/tpl2 KO hepatocytes following incubation for 18 h with APAP plus TNFα (Fig. 4*B*). Furthermore, in APAP plus TNFα-treated hepatocytes Cot/tpl2 deficiency did not modulate the activity of JNK (Fig. 4*C*).

Involvement of Cot/tpl2 in Macrophage Activation by DAMPs—We evaluated the role of Cot/tpl2 in the activation of thioglycolate-elicited peritoneal macrophages by DAMPs obtained from Wt necrotic hepatocytes. After DAMPs stimulation, a decrease in IL-10, IL-1α, IL-1β, IL-6, and TNFα was detected in the supernatant of Cot/tpl2 KO with respect to Wt peritoneal macrophages (Fig. 5*A*). When the activation state of different intracellular pathways was analyzed in thioglycolate-elicited peritoneal macrophages stimulated with DAMPs, p105 NFκB phosphorylation, an upstream effector of Cot/tpl2, was similarly increased in both Wt and Cot/tpl2 KO macrophages (Fig. 5*B*). Moreover, in Wt macrophages, we observed the degradation of both the high and low molecular weight forms of Cot/tpl2, suggesting that it had dissociated from the p105 NFκB-ABIN2-Cot/tpl2 complex (26). Stimulation of both Wt and Cot/tpl2 KO macrophages resulted in an increase in Erk1/2 phosphorylation over 7 min that decreased rapidly in Cot/tpl2-deficient cells. In Wt peritoneal macrophages but not in Cot/tpl2 KO cells, a second increase of P-Erk1/2 levels was observed 30 min after stimulation (Fig. 5*B*). This early Erk1/2 activation peak following macrophage stimulation with DAMPs was not due to a possible FBS contamination contained in the DAMPs (supplemental Fig. S3). Activation of different TLRs in Wt and Cot/tpl2 KO peritoneal macrophages, as described previously in BMDM (46), triggered the phosphorylation of p105 NFκB by IKKβ, whereas Erk1/2 phosphorylation was observed only in Wt macrophages, evident as a single activation peak (supplemental Fig. S4). Cot/tpl2 deficiency decreased JNK activation, but not p38α, in peritoneal macrophages stimulated with Wt DAMPs (Fig. 5*B*). The loss of Cot/tpl2 expression in Cot/tpl2 KO hepatocytes did not affect the capacity of the obtained DAMPs to phosphorylate p105 NFκB or the different MAP kinases, as observed when BMDM were stimulated with Wt or

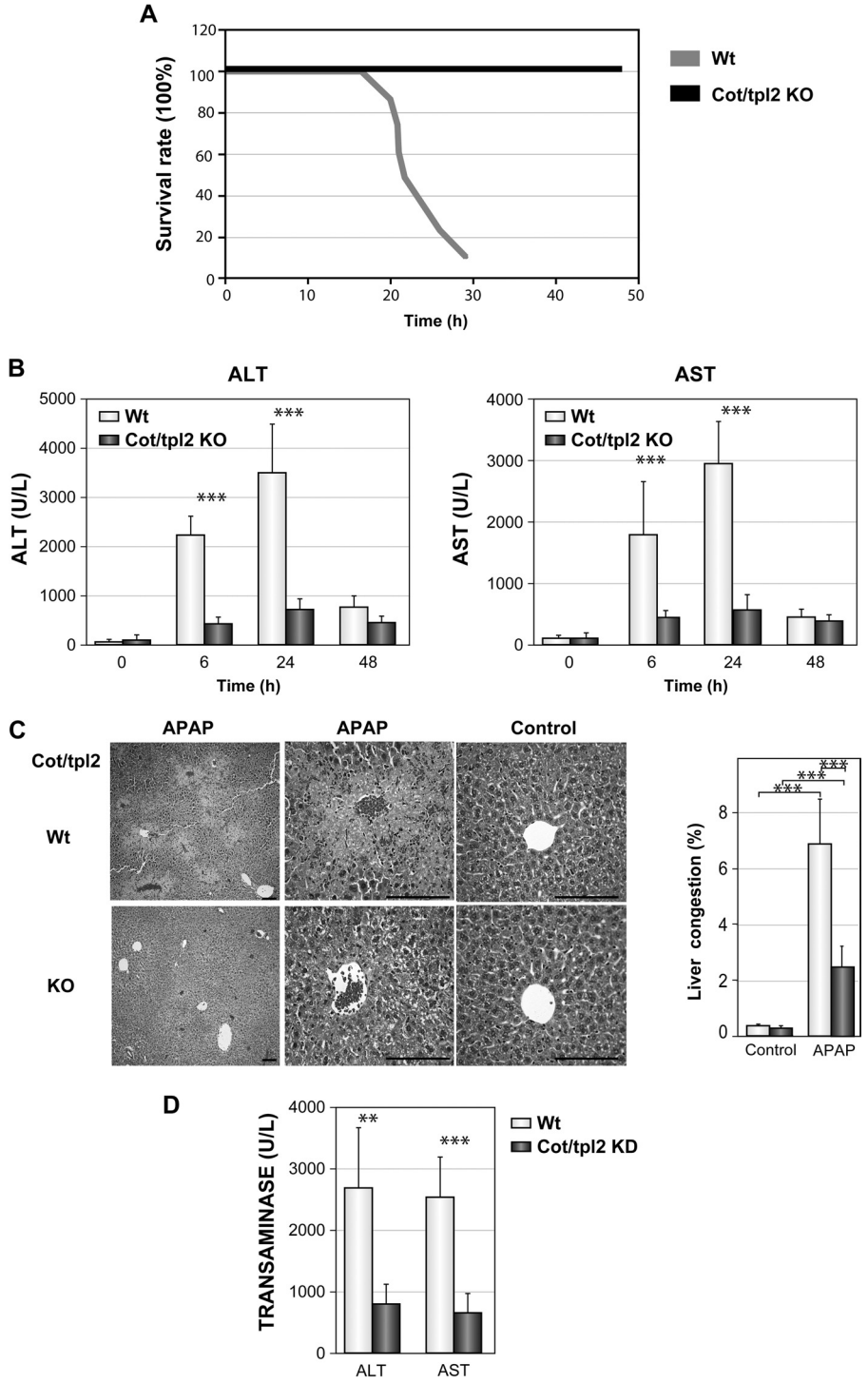


FIGURE 1. **APAP-induced liver injury in Wt and Cot/tpl2-deficient mice.** *A*, survival of Wt ($n = 8$) and Cot/tpl2 KO mice ($n = 8$) after APAP administration (600 g/kg, intraperitoneal). *B*, Cot/tpl2 KO mice were injected with APAP (450 g/kg, intraperitoneal), and serum ALT and AST levels were measured at various times. Data represent the mean ± S.D. from four experiments performed in quadruplicate. *C*, Wt and Cot/tpl2 KO mice were injected (450 g/kg, intraperitoneal) with APAP or with PBS for 24 h. H&E staining of liver sections. Images from PBS-injected mice are representative of three mice, and those from mice that received APAP are representative of seven animals from which 7 randomly selected fields were analyzed. Scale bars, 100 μm. Graph shows the mean ± S.E. from the % congestive area analyzed in 49 different fields obtained from 7 APAP-treated Wt, 7 APAP-treated Cot/tpl2 KO mice, and from 3 WT PBS-injected and 3 Cot/tpl2 KO PBS-injected mice. *D*, Wt and Cot/tpl2 KD mice were injected with APAP (450 g/kg, intraperitoneal), serum ALT and AST levels were determined 18 h later. Graphs represent the mean ± S.D. of three independent experiments performed in quadruplicate.

Cot/tpl2 KO DAMPs (supplemental Fig. S5). As detected in peritoneal macrophages (Fig. 5*B*), the deficient expression of Cot/tpl2 in BMDM abolished the 30–40 min Erk1/2 activation and decreased JNK phosphorylation following the stimulation with Wt or Cot/tpl2 KO DAMPs (Fig. 5*C* and supplemental Fig. S5). Furthermore, in Wt BMDM stimulated with DAMPs, the IKKβ inhibitor B1605906 blocked the activation of Erk1/2 at 40 min but not that at 5 min (Fig. 5*D*). We previously demon-

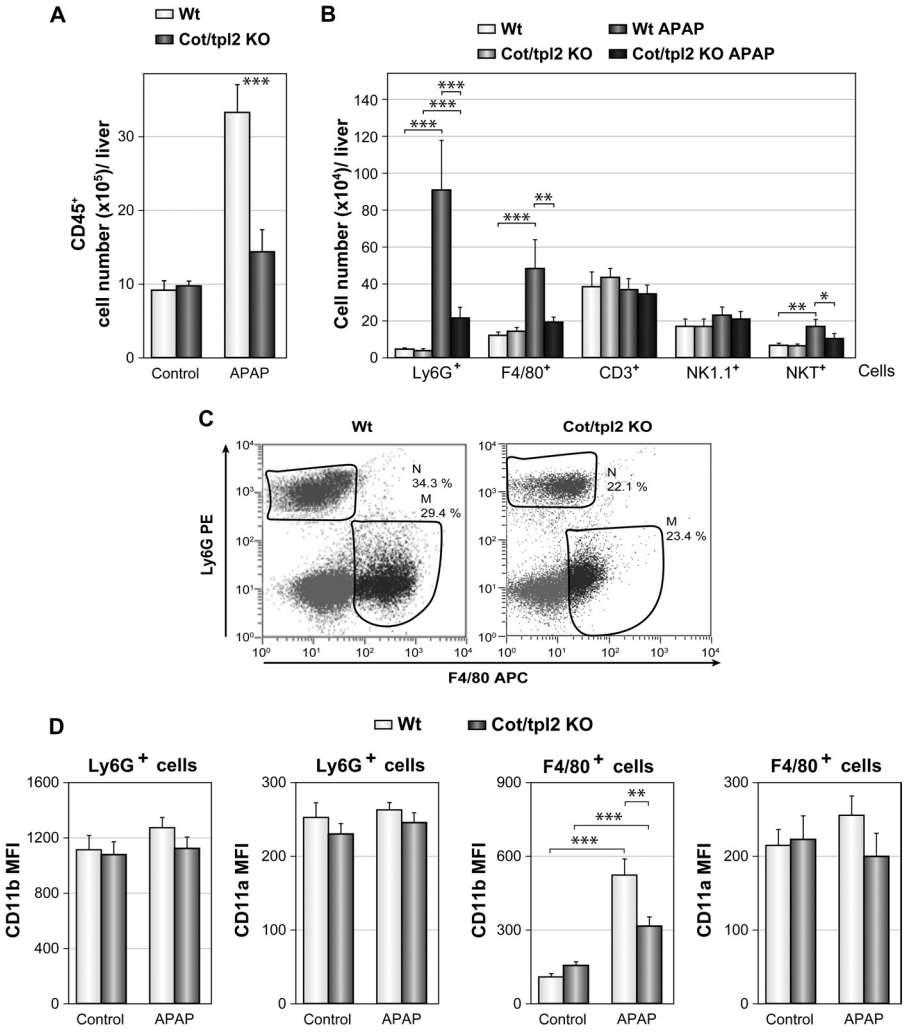


FIGURE 2. **Leukocyte recruitment in the liver of Wt and Cot/tpl2 KO mice following APAP injection.** *A*, mice were injected intraperitoneally with PBS (Control) or APAP (450 g/kg), and CD45$^+$ liver cells were isolated 18 h later and analyzed by flow cytometry. *B*, analysis of Ly6G$^+$, F4/80$^+$, CD3$^+$ (CD3$^+$NK1.1$^-$), NK1.1$^+$ (NK1.1$^+$CD3$^-$), and NKT (CD3$^+$NK1.1$^+$) cells from the CD45$^+$-gated cells in *A*. *C*, representative Ly6G$^+$ *versus* F4/80$^+$ staining FACS profile from the CD45$^+$-gated cells obtained in *A*. *D*, CD11b and CD11a MFI of F4/80$^+$ and Ly6G$^+$ cells isolated from the livers of Wt and Cot/tpl2 KO mice 18 h after intraperitoneal injection with PBS (Control) or APAP (450 mg/kg). Graphs represent the mean ± S.D. of one experiment performed in quadruplicate and similar data were obtained in three additional experiments. Data in *A* and *B* represent the mean ± S.D. of four independent experiments performed in quadruplicate.

strated that Erk1/2 activation by LPS is entirely dependent on Cot/tpl2, whereas Erk1/2 activation by PMA is Cot/tpl2-independent (24). Indeed, IKKβ inhibition blocked LPS- but not PMA-induced Erk1/2 activation (supplemental Fig. S6). Together, these data indicate that two distinct intracellular signal pathways regulate Erk1/2 activation upon DAMPs stimulation of macrophages, one IKKβ-Cot/tpl2-dependent and another IKKβ-Cot/tpl2-independent. Furthermore, taking into account that Cot/tpl2 deficiency impaired both Erk1/2 and JNK phosphorylation in DAMPs-stimulated macrophages, we next decided to investigate the role of both Erk1/2 and JNK MAP kinases in the production of different cytokines. To this end, Wt peritoneal macrophages were stimulated for 18 h with DAMPs in the presence of the JNK inhibitor SP 600125 or in the presence of the Erk1/2 inhibitor PD 0325901. The measurement of IL-10, IL-1α, IL-1β, IL-6, and TNFα levels in the different cell supernatants suggested that Cot/tpl2 mainly controls the production of the different cytokines by its capacity to mediate Erk1/2 activation in DAMPs-stimulated macrophages (supplemental Fig. S7).

Cot/tpl2 Modulates Sterile Inflammation in Vivo—Intraperitoneal injection of DAMPs induces the recruitment of leukocytes to the peritoneum (12, 13). Cot/tpl2 deficiency in mice significantly decreased the peritoneal recruitment of both macrophages and neutrophils 18 h after intraperitoneal injection of Wt or Cot/tpl2 KO DAMPs (Fig. 6, *A* and *B*). However, Cot/tpl2 deficiency did not alter the CD11a and Cd11b MFI values of the cells recruited (supplemental Fig. S8). Similarly, compared with Wt mice, Cot/tpl2 KD mice showed reduced peritoneal recruitment of both macrophages and neutrophils 18 h after intraperitoneal injection of Wt liver homogenate (supplemental Fig. S9). Oxidative burst analysis of the Wt and Cot/tpl2 KO peritoneal leukocytes recruited indicated that Cot/tpl2 deficiency slightly reduced their oxidative burst capacity, a difference that augmented in Cot/tpl2 KO leukocytes upon PMA stimulation (Fig. 6*C*). On the other hand, following intraperitoneal injection of Wt DAMPs, peritoneal levels of IL-1α, IL-1β, and IL-6 diminished in Cot/tpl2 KO mice compared with their Wt counterparts (Fig. 6*D*).

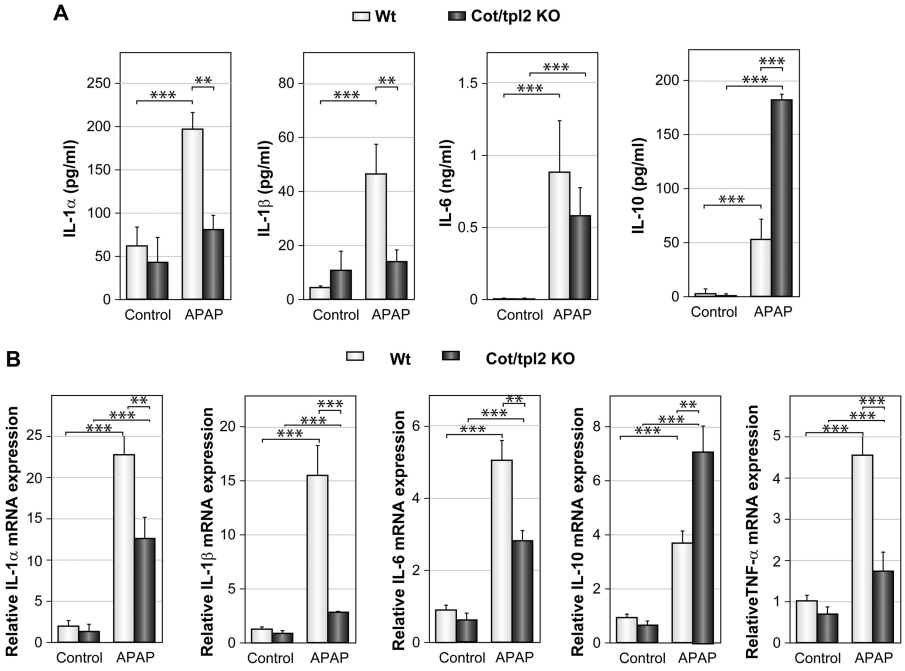


FIGURE 3. **Cot/tpl2 modulates cytokine production associated with APAP-induced liver injury.** Wt and Cot/tpl2 KO mice were challenged with APAP (450 g/kg, intraperitoneal), and cytokines levels were determined. *A*, serum levels of IL-1α, IL-1β, IL-6, and IL-10 were determined 18 h later using a Luminex assay. TNFα could not be detected. *B*, IL-1α, IL-1β, IL-6, IL-10, and TNFα mRNA expression levels upon 6 h of APAP challenge were determined by RT-PCR analysis. *A* and *B*, data represent the mean ± S.D. of four independent experiments performed in quadruplicate.

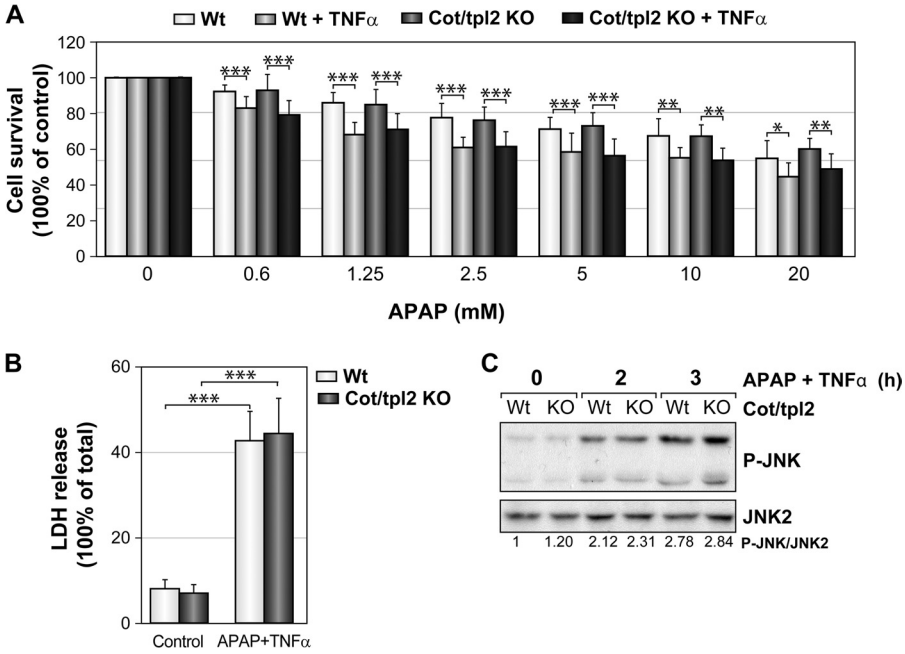


FIGURE 4. **Toxicity induced by APAP plus TNFα in Wt and Cot/tpl2 KO hepatocytes.** *A*, Wt and Cot/tpl2 KO hepatocytes were incubated with 10 ng/ml of TNFα in combination with different concentrations of APAP, and their viability was assessed 18 h later through a MTT assay. The value obtained for Wt and Cot/tpl2 KO hepatocytes incubated in the absence of APAP was considered as 100%. *B*, LDH was determined in the cell supernatant of Wt and Cot/tpl2 KO hepatocytes incubated with 10 ng/ml of TNFα plus 20 mM of APAP for 18 h. The 100% value is given to the one obtained after Triton X-100 permeabilization. *C*, hepatocytes were incubated with 20 mM APAP plus 20 ng/ml TNFα for 0, 2, and 3 h, and P-JNK and JNK2 levels were measured by Western blot. The relative relation of P-JNK levels/JNK2 levels was determined by densitometric quantification of the radiographs, given the value of 1 to the one obtained with Wt hepatocytes at time 0. Similar data have been obtained in three independent experiments. *A* and *B*, graphs show the mean ± S.D. of three independent experiments performed in triplicate.

DISCUSSION

APAP overdoses are the number 1 cause of acute liver failure in the Western world, accounting for 50% of them (1–3). The present study demonstrates the fundamental role of Cot/tpl2 activity in APAP-induced liver damage. APAP-induced liver injury is associated with sterile inflammation (8, 44, 45), and Cot/tpl2 participates in its development by modulating the production of cytokines and the liver recruitment of leukocytes. In this context, it has been previously shown that TLR9 KO mice, with reduced levels of inflammatory cytokines, show resistance

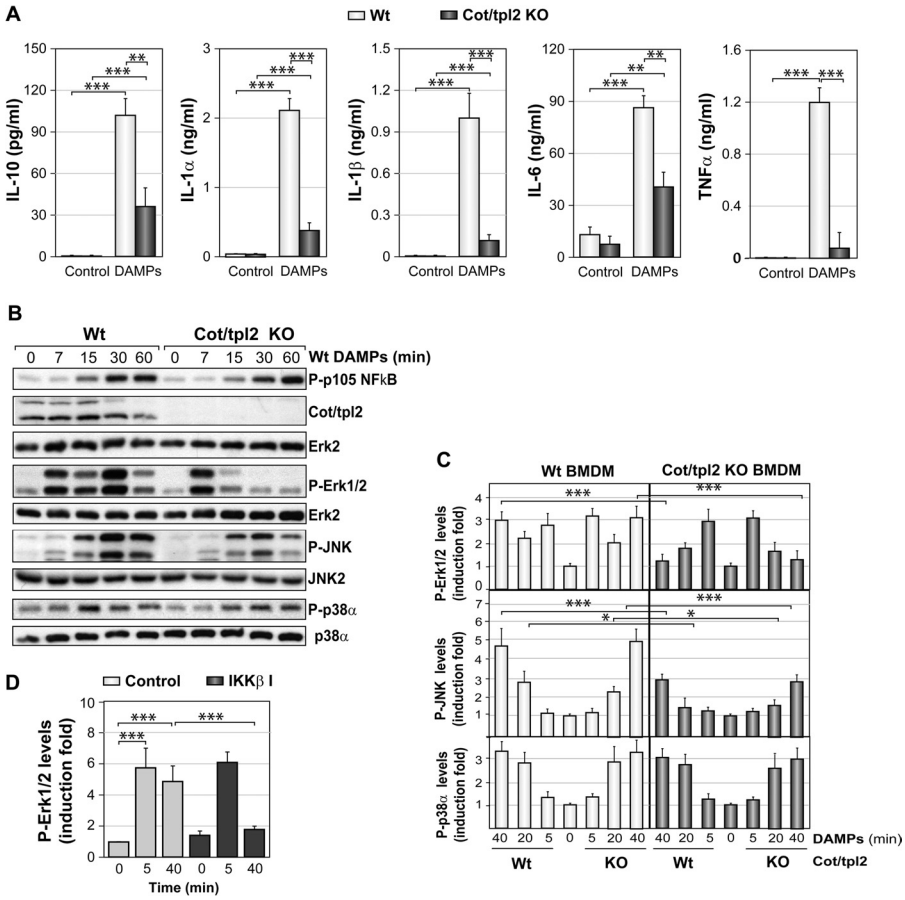


FIGURE 5. **Activation of Wt and Cot/tpl2 KO macrophages by DAMPs.** *A*, concentration of different cytokines in the supernatant of Wt and Cot/tpl2 KO peritoneal macrophages incubated for 18 h in the presence or absence of Wt DAMPs (300 μg/ml). The data represent the results of three independent experiments performed in triplicate. *B*, Wt and Cot/tpl2 KO peritoneal macrophages were treated as described in *A*, and at the times indicated, the levels of P-p105 NFκB, Cot/tpl2, P-Erk1/2, P-JNK, and P-p38α were measured in Western blots. The total Erk2, JNK2, and p38α levels were determined as protein loading controls, and one representative experiment of the three performed is shown. *C*, Wt and Cot/tpl2 KO BMDM were stimulated with Wt and Cot/tpl2 KO DAMPs (300 μg/ml), and at the times indicated, the cell extracts were analyzed in Western blots probed with the antibodies indicated in *B*. The P-p105 NFκB/Erk2, P-Erk1/2/Erk2, P-JNK/JNK2, and P-p38α/p38α values are expressed relative to the Wt time 0 point, and the data in the graphs represent the mean $\pm$ S.D. from four independent experiments. *D*, Wt BMDM were stimulated with Wt DAMPs (300 μg/ml) in the absence (Control) or presence of 10 μM of the IKKβ inhibitor (IKKβ I) B1605906 for the indicated times. Subsequently P-Erk1/2 and Erk2 levels were determined by Western blot analysis. The graphs represent the mean $\pm$ S.D. from three independent experiments of P-Erk1/2 fold induction relative to the Wt time 0 point, after normalizing values to, respectively, total Erk2.

to acetaminophen toxicity (47). While some controversy surrounds the role of macrophages in the pathogenesis of APAP-mediated liver damage, neutrophil recruitment has been implicated in this process (40, 48–50). Indeed, neutrophil infiltration is associated with increased tissue necrosis because of the release of cytotoxic agents such as reactive oxygen species (51).

APAP overdose generates in hepatocytes the *N*-acetyl-p-benzoquinone imine that depletes glutathione and then generates oxidative stress reactions, leading to hepatocyte necrosis (4). DAMPs, once released into the surrounding tissue as a consequence of cell necrosis, orchestrate an inflammatory response, which in many cases can cause a second wave of destruction contributing to the pathogenesis of many damaging conditions (7, 11, 14). DAMPs are recognized by different receptors of the PPR family, including different TLRs (7, 10, 16). However, the i.p. recruitment of inflammatory cells upon intraperitoneal injection of DAMPs is not affected by the loss of a single TLR (12), although the inflammatory response to DAMPs is significantly attenuated in IL-1R and MyD88 KO mice (12). Here, we show that Cot/tpl2 participates in the i.p. recruitment of leukocytes in response to stimulation with DAMPs. IL-1R and virtually all TLRs (except TLR3) signal via MyD88 (20). Indeed, Cot/tpl2, activated via MyD88 or Trif, triggers the MKK1-Erk1/2 pathway following stimulation of IL-1R and TNFαR and also of all the different TLRs, including TLR3 (29, 30, 46).

Here we show that Cot/tpl2 once activated mediates, both *in vivo* and *in vitro*, IL-1α and IL-1β production. Sterile inflammation is largely dependent on IL-1α. Indeed, sterile inflammation requires this cytokine to a greater extent than pathogen-induced inflammation and its role in sterile inflammation cannot be substituted by IL-1β or any other cytokine (52–55). The peritoneal recruitment of neutrophils in response to DAMPs is largely dependent on IL-1α (13), and it has been recently reported that IL-1α and IL-1β trigger the recruitment of different myeloid cells (53).

In LPS-stimulated macrophages Cot/tpl2 mediates the production of the pro-inflammatory cytokine TNFα, but also of the anti-inflammatory cytokine IL-10 (25, 42, 56), and similar effects were observed here upon stimulation of macrophages with DAMPs. However, the contribution of Cot/tpl2 to the expression of these cytokines *in vivo* is not as straightforward as

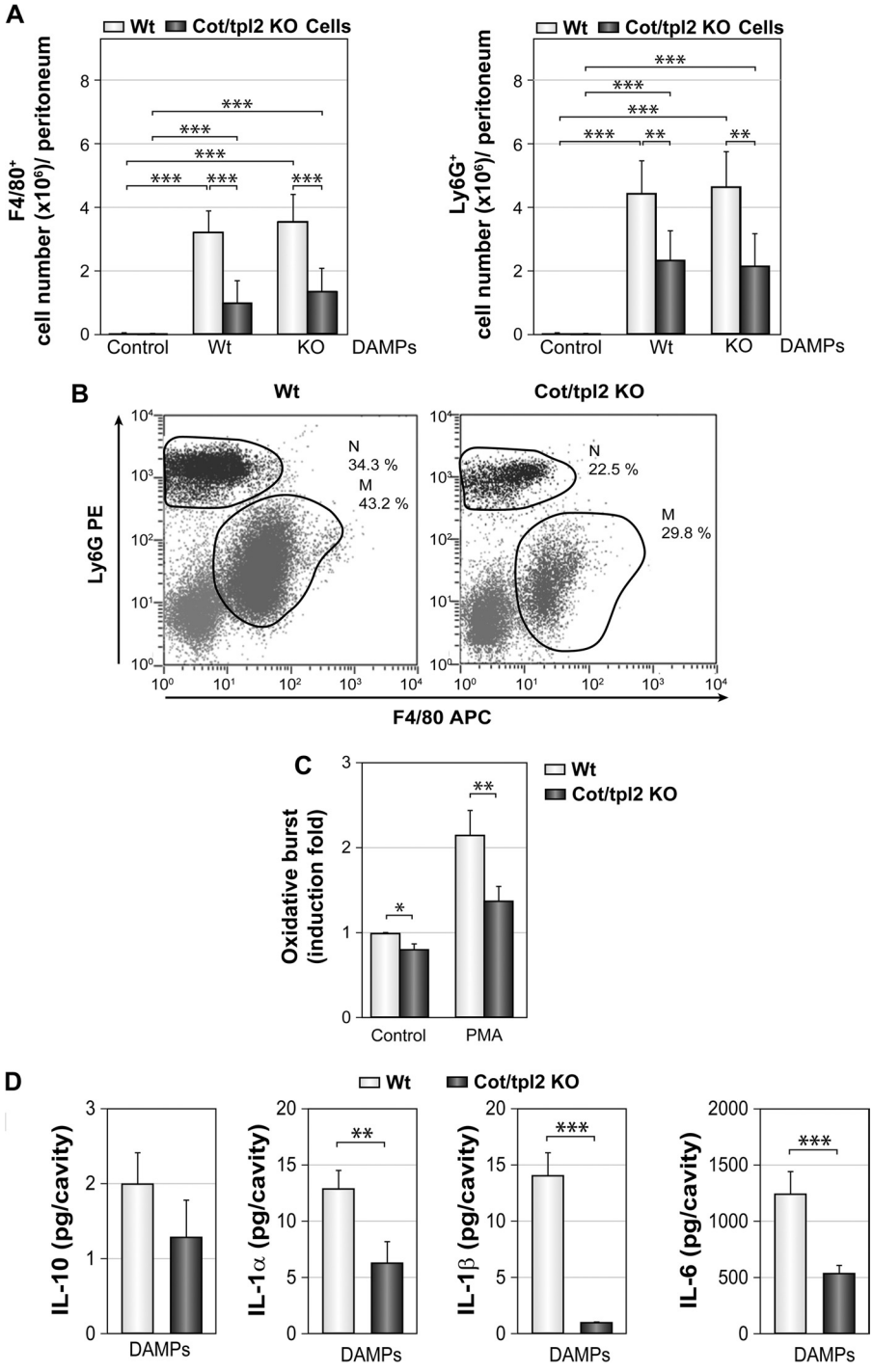


FIGURE 6. **Leukocytes recruitment following DAMPs-induced peritonitis in Wt and Cot/tpl2 KO mice.** Animals were injected (intraperitoneal) with Wt or Cot/tpl2 KO DAMPs (1.7 mg) or with PBS (Control), and peritoneal cells were isolated 18 h later. *A*, number of isolated peritoneal macrophages (F4/80$^+$) and neutrophils (LY6G$^+$) recovered after injection of Wt or Cot/tpl2 KO DAMPs in Wt and Cot/tpl2 KO mice. *B*, one representative Ly6G$^+$ *versus* F4/80$^+$ staining FACS profile of Wt and Cot/tpl2 KO peritoneal cells isolated 18 h after injection of Wt DAMPs in Wt and Cot/tpl2 KO mice. *C*, oxidative burst activity of isolated Wt and Cot/tpl2 KO peritoneal cells incubated in the presence or absence of PMA (10 μM) *in vitro*. The relative induction is expressed in terms of that obtained in unstimulated Wt cells. *D*, levels of the indicated cytokines in the peritoneal cavity of Wt and Cot/tpl2 KO mice treated as described in *B*. *A*, *C*, and *D*, graphs represent the mean ± S.D. of three independent experiments performed in triplicate.

that observed in individual isolated cells. Cot/tpl2 controls TNFα synthesis upon i.p. injection of LPS/D-galactosamine in mice (25) but not following zymosan-induced intraplantar inflammation or following an infection with Listeria monocytogenes (34, 57). Conversely, Cot/tpl2 deficiency in APC KO mice results in a decrease in serum IL-10 (58), while in LPS and CpG-DNA-treated mice, Cot/tpl2 blocks IL-10 production (59). Our findings demonstrate that Cot/tpl2 only mildly increases peritoneal IL-10 levels upon injection with DAMPs, and it decreases serum IL-10 levels in response to APAP-induced liver injury. Although IL-10 protects against APAP-induced liver injury (60), it remains to be determined whether the

increased levels of IL-10 in Cot/tpl2 KO mice at least in part attenuate liver injury.

Tissue damage is recognized at the cell level. Different DAMPs are recognized by the different receptors of the PPR family. The failure of DAMPs from necrotic cells to express specific molecules modifies their capacity to develop sterile inflammation (7, 15–18). However, dampened Cot/tpl2 expression in hepatocytes does not affect the capacity of their obtained DAMPs to activate macrophages *in vitro* or to initiate the sterile inflammation response *in vivo*.

Sterile inflammation is initiated by DAMPs that upon stimulation of different cell types including macrophages (12), produce IL-1α a major regulator of sterile inflammation (52–55). In macrophages, DAMPs activate Erk1/2, and our data indicate that Cot/tpl2 mediates the activation of Erk1/2 after 30 min but not after 5 min following stimulation. This rapid increase in Erk1/2 phosphorylation is probably mediated by the activation of another MAP3K, most likely one of the RAF proteins. Cot/tpl2 is the only MAP3K to activate the Erk1/2 pathway in response to both TLR activation and IL-1 or TNFα stimulation (29, 30). However, cell stimulation with DAMPs results in activation of a variety of receptors (7), thus the two different intracellular pathways involved in Erk1/2 phosphorylation under this cellular condition. Cot/tpl2 can also activate JNK (22, 30), and here we show that Cot/tpl2 partially mediates JNK phosphorylation upon macrophage activation with DAMPs. However, the decreased production of cytokines in the macrophages from Cot/tpl2 KO mice is due to impaired activation of the Erk1/2 intracellular signal pathway. In conclusion, our data show that Cot/tpl2 participates in sterile inflammatory pathways triggered by damaged tissue and plays an essential role in APAP-induced liver injury.

Acknowledgments—B1605906, SP 600125, and PD 0325901 were gifts from Sir Philip Cohen (Dundee).

REFERENCES

1. Kaufman, D. W., Kelly, J. P., Rosenberg, L., Anderson, T. E., and Mitchell, A. A. (2002) Recent patterns of medication use in the ambulatory adult population of the United States: the Slone survey. *JAMA* **287,** 337–344
2. Blazer, D. G., and Wu, L. T. (2009) Nonprescription use of pain relievers by middle-aged and elderly community-living adults: National Survey on Drug Use and Health. *J. Am. Geriatr. Soc.* **57,** 1252–1257
3. Wilcox, C. M., Cryer, B., and Triadafilopoulos, G. (2005) Patterns of use and public perception of over-the-counter pain relievers: focus on nonsteroidal antiinflammatory drugs. *J. Rheumatol.* **32,** 2218–2224
4. Hinson, J. A., Roberts, D. W., and James, L. P. (2010) Mechanisms of acetaminophen-induced liver necrosis. *Handb. Exp. Pharmacol.* **196,** 369–405
5. Jones, D. P., Lemasters, J. J., Han, D., Boelsterli, U. A., and Kaplowitz, N. (2010) Mechanisms of pathogenesis in drug hepatotoxicity putting the stress on mitochondria. *Mol. Interv.* **10,** 98–111
6. McCall, K. (2010) Genetic control of necrosis - another type of programmed cell death. *Curr. Opin. Cell Biol.* **22,** 882–888
7. Chen, G. Y., and Nunez, G. (2010) Sterile inflammation: sensing and reacting to damage. *Nat. Rev. Immunol.* **10,** 826–837
8. Adams, D. H., Ju, C., Ramaiah, S. K., Uetrecht, J., and Jaeschke, H. (2010) Mechanisms of immune-mediated liver injury. *Toxicol. Sci.* **115,** 307–321
9. Rock, K. L., Latz, E., Ontiveros, F., and Kono, H. (2010) The sterile inflammatory response. *Annu. Rev. Immunol.* **28,** 321–342
10. Takeuchi, O., and Akira, S. (2010) Pattern recognition receptors and inflammation. *Cell* **140,** 805–820
11. Maher, J. J. (2009) DAMPs ramp up drug toxicity. *J. Clin. Invest.* **119,** 246–249
12. Kono, H., Karmarkar, D., Iwakura, Y., and Rock, K. L. (2010) Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. *J. Immunol.* **184,** 4470–4478
13. Chen, C. J., Kono, H., Golenbock, D., Reed, G., Akira, S., and Rock, K. L. (2007) Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. *Nat. Med.* **13,** 851–856
14. Stirnimann, G., Kessebohm, K., and Lauterburg, B. (2010) Liver injury caused by drugs: an update. *Swiss Med. Wkly* **140,** w13080
15. Kono, H., and Rock, K. L. (2008) How dying cells alert the immune system to danger. *Nat. Rev. Immunol.* **8,** 279–289
16. Piccinini, A. M., and Midwood, K. S. (2010) DAMPening inflammation by modulating TLR signalling. *Mediators Inflamm.* **2010**
17. Bianchi, M. E. (2007) DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of Leukocyte Biology* **81,** 1–5
18. Sirisinha, S. (2011) Insight into the mechanisms regulating immune homeostasis in health and disease. *Asian Pac. J. Allergy Immunol.* **29,** 1–14
19. Schwabe, R. F., Seki, E., and Brenner, D. A. (2006) Toll-like receptor signaling in the liver. *Gastroenterology* **130,** 1886–1900
20. O'Neill, L. A., and Bowie, A. G. (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signaling. *Nat. Rev. Immunol.* **7,** 353–364
21. Akira, S., and Takeda, K. (2004) Toll-like receptor signalling. *Nat. Rev. Immunol.* **4,** 499–511
22. Gantke, T., Sriskantharajah, S., Sadowski, M., and Ley, S. C. (2012) IκB kinase regulation of the TPL-2/ERK MAPK pathway. *Immunol. Rev.* **246,** 168–182
23. Vougioukalaki, M., Kanellis, D. C., Gkouskou, K., and Eliopoulos, A. G. (2011) Tpl2 kinase signal transduction in inflammation and cancer. *Cancer Lett.* **304,** 80–89
24. Caivano, M., Rodriguez, C., Cohen, P., and Alemany, S. (2003) 15-Deoxy-Δ12,14-prostaglandin J2 regulates endogenous Cot MAPK kinase kinase 1 activity induced by lipopolysaccharide. *J. Biol. Chem.* **278,** 52124–52130
25. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., and Tsichlis, P. N. (2000) TNF-α induction by LPS is regulated post-transcriptionally via a Tpl2/ERK-dependent pathway. *Cell* **103,** 1071–1083
26. López-Peláez, M., Soria-Castro, I., Boscá, L., Fernández, M., and Alemany, S. (2011) Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. *Eur. J. Immunol.* **41,** 1733–1741
27. Waterfield, M. R., Zhang, M., Norman, L. P., and Sun, S. C. (2003) NF-κB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. *Mol. Cell* **11,** 685–694
28. Gándara, M. L., López, P., Hernando, R., Castaño, J. G., and Alemany, S. (2003) The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. *Mol. Cell Biol.* **23,** 7377–7390
29. Rodríguez, C., Pozo, M., Nieto, E., Fernández, M., and Alemany, S. (2006) TRAF6 and Src kinase activity regulates Cot activation by IL-1. *Cell Signal* **18,** 1376–1385
30. Das, S., Cho, J., Lambertz, I., Kelliher, M. A., Eliopoulos, A. G., Du, K., and Tsichlis, P. N. (2005) Tpl2/cot signals activate ERK, JNK, and NF-κB in a cell-type and stimulus-specific manner. *J. Biol. Chem.* **280,** 23748–23757
31. Perugorria, M. J., Murphy, L. B., Fullard, N., Chakraborty, J. B., Vyrla, D., Wilson, C. L., Oakley, F., Mann, J., and Mann, D. A. (2013) Tumor progression locus 2/Cot is required for activation of extracellular regulated kinase in liver injury and toll-like receptor-induced TIMP-1 gene transcription in hepatic stellate cells in mice. *Hepatology* **57,** 1238–1349
32. Cohen, P. (2009) Targeting protein kinases for the development of anti-inflammatory drugs. *Curr. Opin. Cell Biol.* **21,** 317–324
33. Gaestel, M., Kotlyarov, A., and Kracht, M. (2009) Targeting innate immunity protein kinase signalling in inflammation. *Nat. Rev. Drug Discov.* **8,** 480–499
34. Soria-Castro, I., Krzyzanowska, A., Pelaéz, M. L., Regadera, J., Ferrer, G., Montoliu, L., Rodríguez-Ramos, R., Fernández, M., and Alemany, S.

(2010) Cot/tpl2 (MAP3K8) mediates myeloperoxidase activity and hypernociception following peripheral inflammation. *J. Biol. Chem.* **285,** 33805–33815
35. Kontoyiannis, D., Boulougouris, G., Manoloukos, M., Armaka, M., Apostolaki, M., Pizarro, T., Kotlyarov, A., Forster, I., Flavell, R., Gaestel, M., Tsichlis, P., Cominelli, F., and Kollias, G. (2002) Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. *J. Exp. Med.* **196,** 1563–1574
36. Koliaraki, V., Roulis, M., and Kollias, G. (2012) Tpl2 regulates intestinal myofibroblast HGF release to suppress colitis-associated tumorigenesis. *J. Clin. Invest.* **122,** 4231–4242
37. Van Acker, G. J., Perides, G., Weiss, E. R., Das, S., Tsichlis, P. N., and Steer, M. L. (2007) Tumor progression locus-2 is a critical regulator of pancreatic and lung inflammation during acute pancreatitis. *J. Biol. Chem.* **282,** 22140–22149
38. Watford, W. T., Wang, C. C., Tsatsanis, C., Mielke, L. A., Eliopoulos, A. G., Daskalakis, C., Charles, N., Odom, S., Rivera, J., O'Shea, J., and Tsichlis, P. N. (2010) Ablation of tumor progression locus 2 promotes a type 2 Th cell response in Ovalbumin-immunized mice. *J. Immunol.* **184,** 105–113
39. González-Rodriguez, A., Escribano, O., Alba, J., Rondinone, C. M., Benito, M., and Valverde, A. M. (2007) Levels of protein tyrosine phosphatase 1B determine susceptibility to apoptosis in serum-deprived hepatocytes. *J. Cell Physiol.* **212,** 76–88
40. Liu, Z. X., Han, D., Gunawan, B., and Kaplowitz, N. (2006) Neutrophil depletion protects against murine acetaminophen hepatotoxicity. *Hepatology* **43,** 1220–1230
41. Rodríguez, C., López, P., Pozo, M., Duce, A. M., López-Pelaéz, M., Fernández, M., and Alemany, S. (2008) COX2 expression and Erk1/Erk2 activity mediate Cot-induced cell migration. *Cell Signal* **20,** 1625–1631
42. López-Pelaéz, M., Fumagalli, S., Sanz, C., Herrero, C., Guerra, S., Fernandez, M., and Alemany, S. (2012) Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor-activated macrophages. *Mol. Biol. Cell* **23,** 2982–2992
43. Gunawan, B. K., Liu, Z. X., Han, D., Hanawa, N., Gaarde, W. A., and Kaplowitz, N. (2006) c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology* **131,** 165–178
44. Holt, M. P., Cheng, L., and Ju, C. (2008) Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. *J. Leukoc Biol.* **84,** 1410–1421
45. Antoniades, C. G., Quaglia, A., Taams, L. S., Mitry, R. R., Hussain, M., Abeles, R., Possamai, L. A., Bruce, M., McPhail, M., Starling, C., Wagner, B., Barnardo, A., Pomplun, S., Auzinger, G., Bernal, W., Heaton, N., Vergani, D., Thursz, M. R., and Wendon, J. (2012) Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. *Hepatology* **56,** 735–746
46. Banerjee, A., Gugasyan, R., McMahon, M., and Gerondakis, S. (2006) Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. *Proc. Natl. Acad. Sci. U.S.A.* **103,** 3274–3279
47. Imaeda, A. B., Watanabe, A., Sohail, M. A., Mahmood, S., Mohamadnejad, M., Sutterwala, F. S., Flavell, R. A., and Mehal, W. Z. (2009) Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. *J. Clin. Invest.* **119,** 305–314
48. Wang, X., Sun, R., Wei, H., and Tian, Z. (2013) High-mobility group box 1 (HMGB1)-Toll-like receptor (TLR)4-interleukin (IL)-23-IL-17A axis in drug-induced damage-associated lethal hepatitis: Interaction of γδ T cells with macrophages. *Hepatology* **57,** 373–384
49. Marques, P. E., Amaral, S. S., Pires, D. A., Nogueira, L. L., Soriani, F. M., Freire Lima, B. H., Oliveira Lopes, G. A., Russo, R. C., Avila, T. V., Melgaço, J. G., Oliveira, A. G., Pinto, M. A., Lima, C. X., de Paula, A. M., Cara, D. C., Leite, M. F., Teixeira, M. M., and Menezes, G. B. (2012) Chemokines and mitochondrial products activate neutrophils to amplify organ injury during mouse acute liver failure. *Hepatology* **56,** 1971–1982
50. Jaeschke, H., McGill, M. R., and Williams, C. D. (2013) Pathophysiological relevance of neutrophils in acetaminophen hepatotoxicity. *Hepatology* **57,** 419
51. Weiss, S. J. (1989) Tissue destruction by neutrophils. *N. Engl. J. Med.* **320,** 365–376
52. Berda-Haddad, Y., Robert, S., Salers, P., Zekraoui, L., Farnarier, C., Dinarello, C. A., Dignat-George, F., and Kaplanski, G. (2011) Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1α. *Proc. Natl. Acad. Sci. U.S.A.* **108,** 20684–20689
53. Rider, P., Carmi, Y., Guttman, O., Braiman, A., Cohen, I., Voronov, E., White, M. R., Dinarello, C. A., and Apte, R. N. (2011) IL-1α and IL-1β recruit different myeloid cells and promote different stages of sterile inflammation. *J. Immunol.* **187,** 4835–4843
54. Eigenbrod, T., Park, J. H., Harder, J., Iwakura, Y., and Nuñez, G. (2008) Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. *J. Immunol.* **181,** 8194–8198
55. Elkon, K. B. (2007) IL-1α responds to necrotic cell death. *Nat. Med.* **13,** 778–780
56. Kaiser, F., Cook, D., Papoutsopoulou, S., Rajsbaum, R., Wu, X., Yang, H. T., Grant, S., Ricciardi-Castagnoli, P., Tsichlis, P. N., Ley, S. C., and O'Garra, A. (2009) TPL-2 negatively regulates interferon-β production in macrophages and myeloid dendritic cells. *J. Exp. Med.* **206,** 1863–1871
57. Mielke, L. A., Elkins, K. L., Wei, L., Starr, R., Tsichlis, P. N., O'Shea, J. J., and Watford, W. T. (2009) Tumor progression locus 2 (Map3k8) is critical for host defense against Listeria monocytogenes and IL-1β production. *J. Immunol.* **183,** 7984–7993
58. Serebrennikova, O. B., Tsatsanis, C., Mao, C., Gounaris, E., Ren, W., Siracusa, L. D., Eliopoulos, A. G., Khazaie, K., and Tsichlis, P. N. (2012) Tpl2 ablation promotes intestinal inflammation and tumorigenesis in Apcmin mice by inhibiting IL-10 secretion and regulatory T-cell generation. *Proc. Natl. Acad. Sci. U.S.A.* **109,** E1082–E1091
59. Sugimoto, K., Ohata, M., Miyoshi, J., Ishizaki, H., Tsuboi, N., Masuda, A., Yoshikai, Y., Takamoto, M., Sugane, K., Matsuo, S., Shimada, Y., and Matsuguchi, T. (2004) A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation. *J. Clin. Invest.* **114,** 857–866
60. Bourdi, M., Masubuchi, Y., Reilly, T. P., Amouzadeh, H. R., Martin, J. L., George, J. W., Shah, A. G., and Pohl, L. R. (2002) Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. *Hepatology* **35,** 289–298

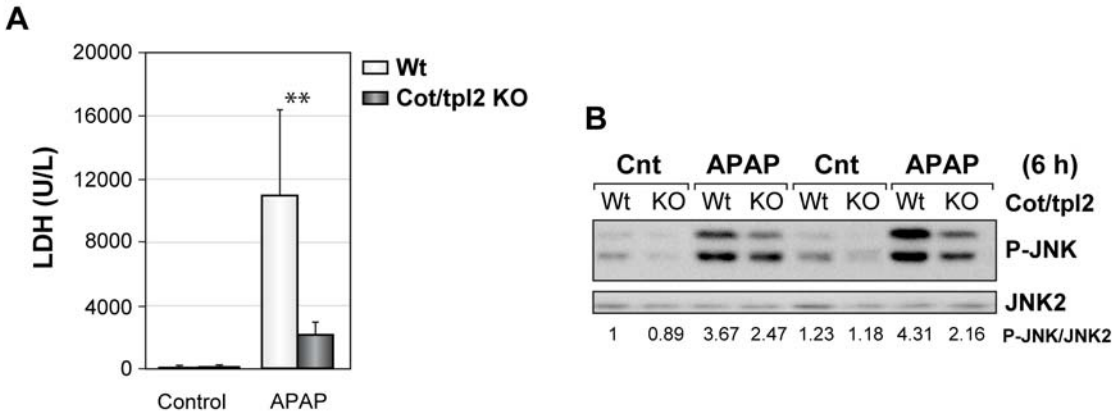


Figure S1. Induction of the liver damage markers LDH and P-JNK in APAP-injected Wt and Cot/tpl2 KO mice. A. Wt and Cot/tpl2 KO mice were i.p. injected with APAP (450 g/kg) and 24 h later serum LDH levels were determined. Data are the mean ± SD from 4 experiments performed in cuatriplicate. B. Western-blot analysis of hepatic P-JNK and total JNK2 levels from Wt and Cot/tpl2 KO mice upon 6 h i.p. injection of APAP (450 g/kg) or PBS (Cnt) was performed. Relative relation of P-JNK levels/ JNK2 levels was determined by densitometric quantification of the radiographs, given the value of 1 to the obtained with one of the control Wt mice. Data shown the results from 2 independent experiments from the 4 performed.

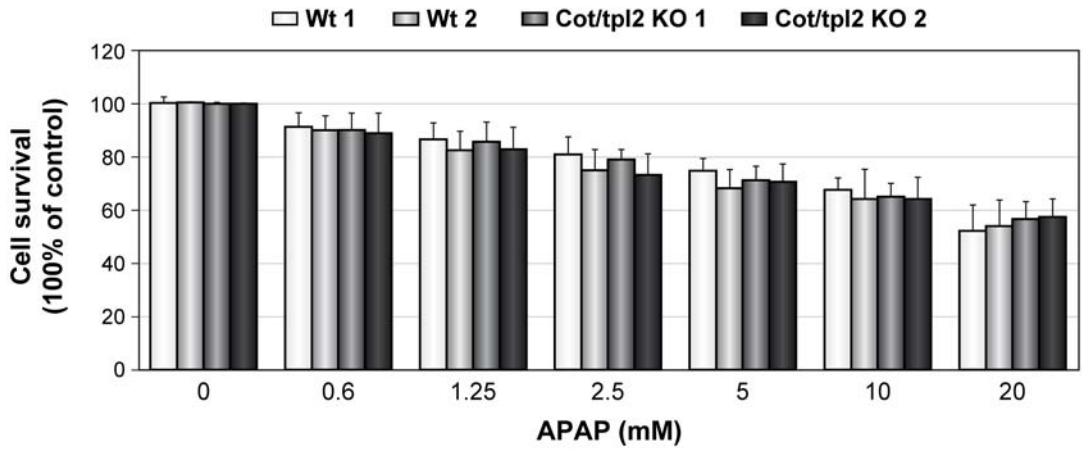


Figure S2. APAP-induced toxicity in Wt and Cot/tpl2 KO hepatocytes. Two different pools of immortalized Wt and Cot/tpl2 KO hepatocytes were incubated with different concentrations of APAP and 18 h later cells were subjected to a MTT viability assay. 100% value is given to Wt and Cot/tpl2 KO hepatocytes incubated in the absence of APAP. Graph show the mean ± SD of 3 experiments performed in triplicate.

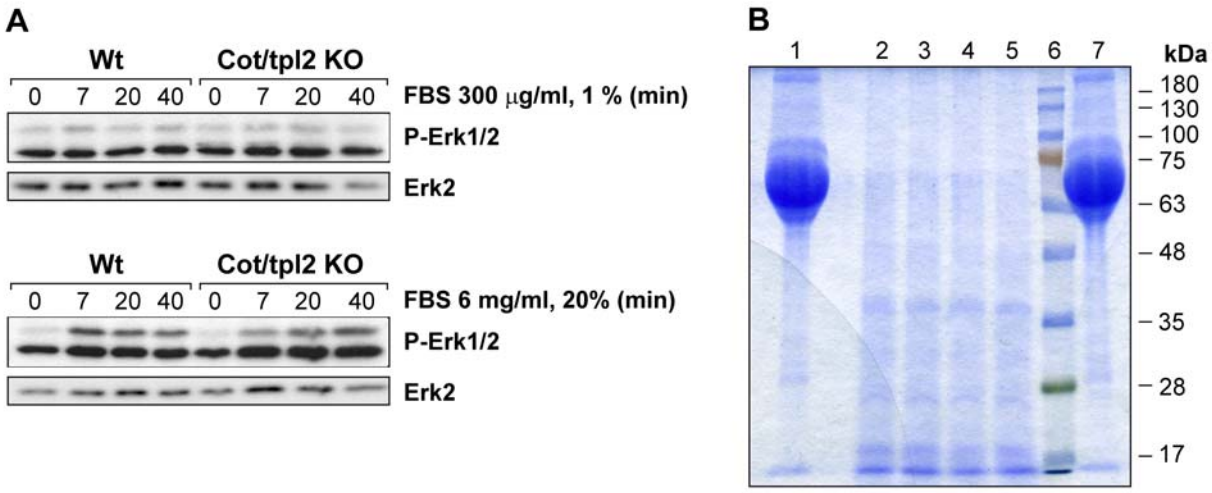


Figure S3. Erk1/2 phosphorylation in Wt and Cot/tpl2 KO BMDM stimulated with FBS. A) Wt and Cot/tpl2 KO BMDM were stimulated with 300 μg/ml (1%) or 6 mg/ml (20 %) of FBS and P-Erk1/2 as well as Erk2 levels were determined by Western-blot. Figures show one representative experiment of the 2 performed in duplicate. **B)** FBS and two different Wt and Cot/tpl2 KO DAMPs preparations were run on a 12% SDS-polyacrylamide gel electrophoresis, subsequently gel was stained with Comassie Blue. Thirty micrograms of FBS (lane 1), 30 μg of Wt DAMPs 1 (lane 2), 30 μg of Wt DAMPs 2 (lane 3), 30 μg of Cot/tpl2 KO DAMPs 1 (lane 4) 30 μg of Cot/tpl2 KO DAMPs 2 (lane 5), protein markers (lane 6), 30 μg of FBS (lane 7).

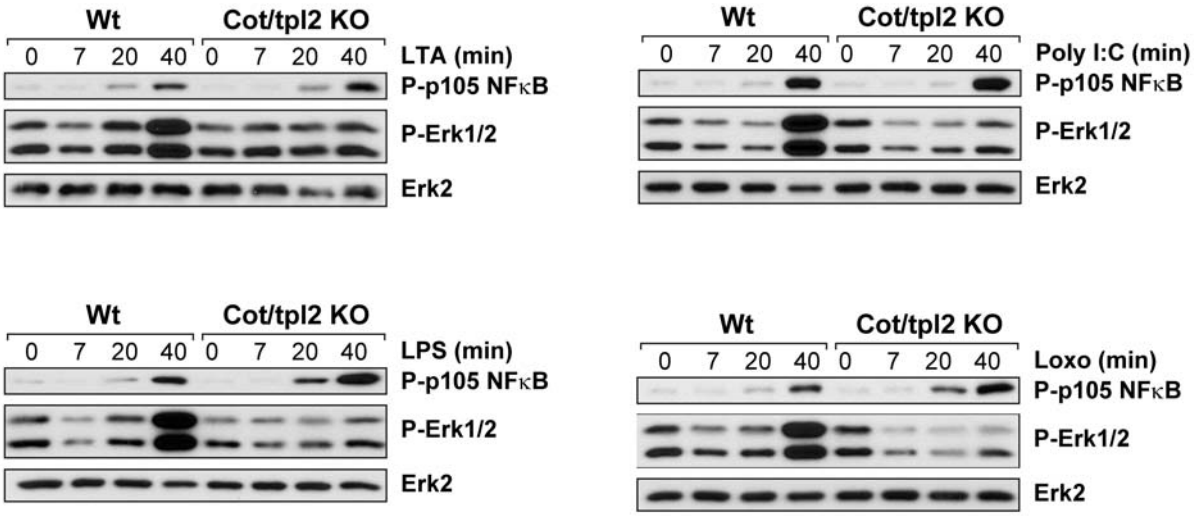


Figure S4. p105 NFκB and Erk1/2 phosphorylation in TLR-activated Wt and Cot/tpl2 KO peritoneal macrophages**.** Wt and Cot/tpl2 KO peritoneal macrophages were stimulated with the TLR2 ligand, Lipoteichoic acid from *Bacillus subtilis* (LTA, 5 μg/ml), the TLR3 ligand Poly I:C at 5 μg/ml, the TLR4 ligand Lipopolysaccharide from *Salmonella typhimurium*, (*LPS, 300 ng/ml*), and the TLR7 ligand, Loxoribine (Loxo, 500 μg/ml). After indicated times P-p105 NFκB and P-Erk1/2 levels were measured by Western-blot. Erk2 expression levels were determined as a protein loading control. Figure shows one representative experiment of the 3 performed.

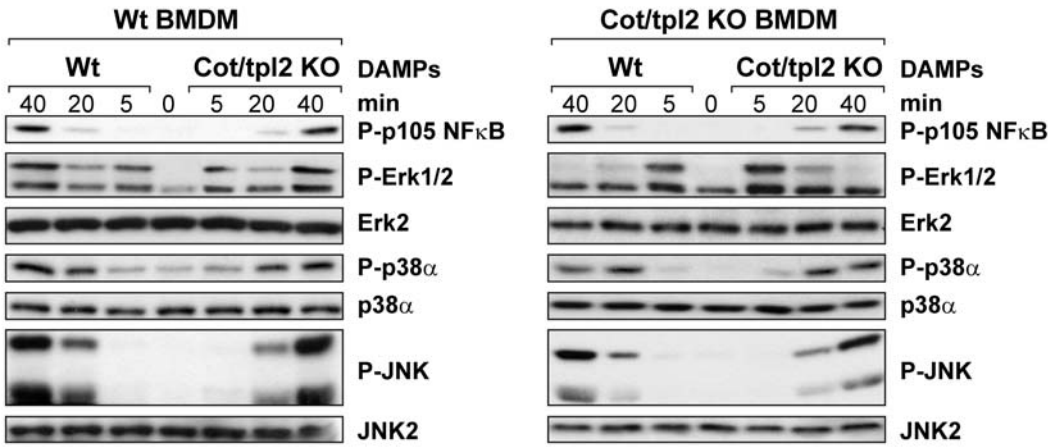


Figure S5. Activation of different MAP kinases in Wt and Cot/tpl2 KO BMDM stimulated with Wt and with Cot/tpl2 KO DAMPs. Wt and Cot/tpl2 KO BMDM were stimulated with Wt or Cot/tpl2 KO DAMPs (300 microgram/ml) for the indicated times. Cell extracts were used for Western-blot analysis to determine P-p105 NFκB, P-Erk1/2, P-JNK, and P-p38α levels. Erk2, JNK2, and p38α were determined as a protein loading control. Figure shows one representative experiment of the 4 performed.

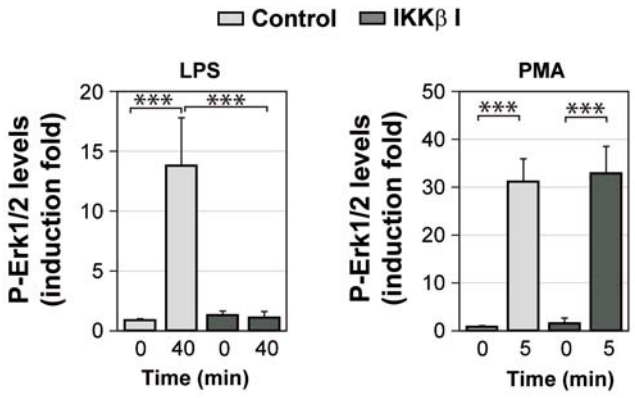


Figure S6. Modulation of Erk1/2 phosphorylation by IKKβ inhibition in BMDM stimulated with LPS and PMA. Wt BMDM were stimulated with LPS (300 ng/ml) or with PMA (10 μg/ml) in the absence (Control) or presence of 10 μmolar of the IKKβ inhibitor (IKKβ I) B1605906 for the indicated times. Subsequently P-Erk1/2 and Erk2 levels were determined by Western-blot analysis. Graphs represent the mean ± SD from 3 independent experiments of P-Erk1/2 fold induction relative to the Wt 0 time point, after normalizing values to respectively total Erk2.

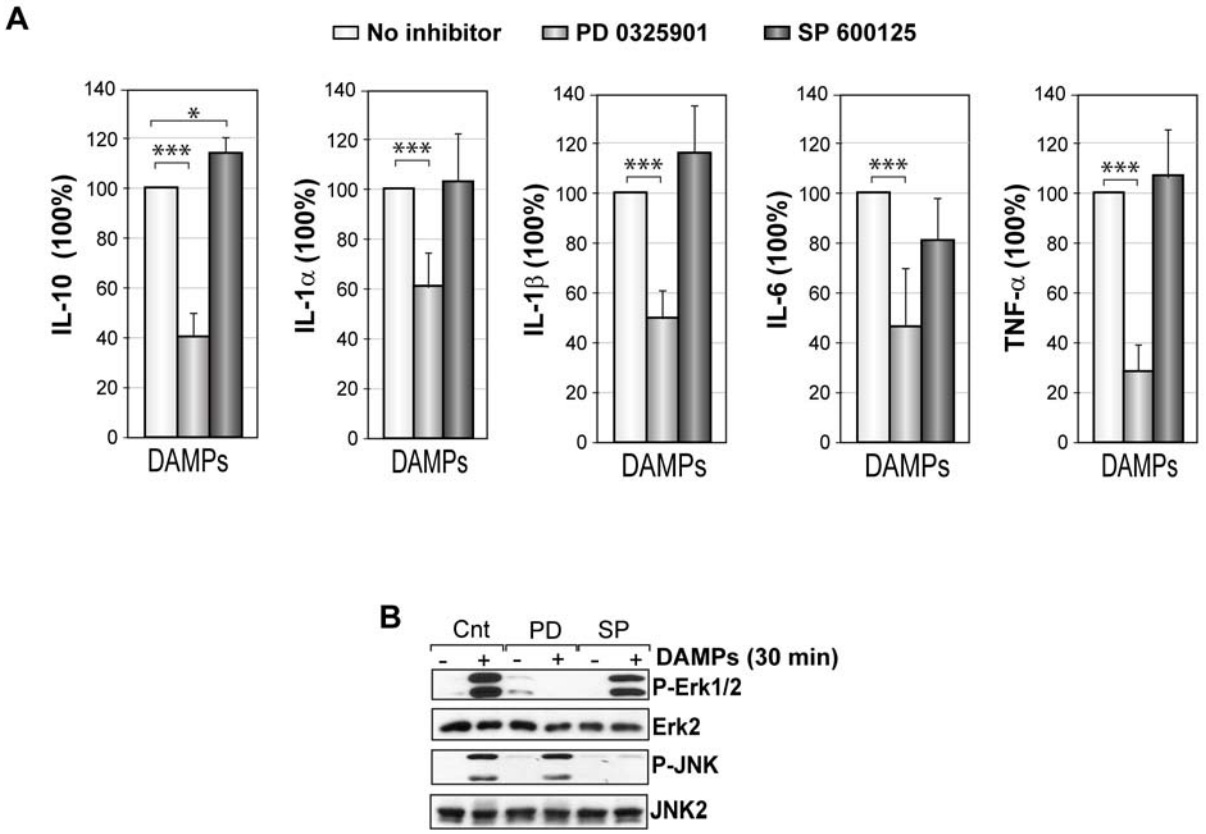


Figure S7. Role of JNK and Erk1/2 activity in the production of cytokines in DAMPs-stimulated macrophages. A. Peritoneal macrophages preincubated or not for 30 min with the JNK inhibitor SP 600125 (12 μmolar) or with the Erk1/2 inhibitor PD 0325901 (0.5 μmolar) were stimulated or not with Wt DAMPs (300 microgram/ml) for 18 h and subsequently the concentration of different cytokines in the cell supernatant was determined. The data represent the results of 3 independent experiments performed in triplicate. The value 100% is given to the value obtained in DAMPs-stimulated macrophages in the absence of any inhibitor B. Wt and Cot/tpl2 KO peritoneal macrophages were treated as described in A, and 30 min following stimulation, the levels P-Erk1/2 and P-JNK were measured in Western blots. The total Erk2 and JNK levels were determined as protein loading controls, and one representative experiment of the 3 performed is shown.

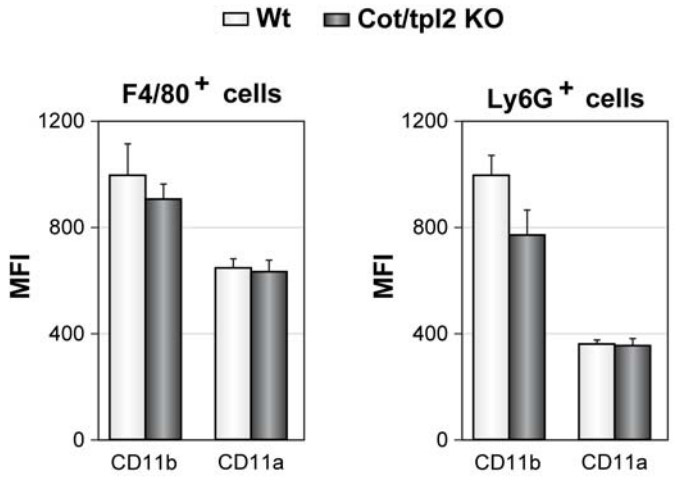


Figure S8. CD11b and CD11a MFI values of F4/80$^+$ and Ly6G$^+$ cells isolated from the peritoneal cavity following an intraperitoneal injection of DAMPs. CD11b and CD11a MFI values from F4/80$^+$ and Ly6G$^+$ cells isolated from the peritoneal cavity of Wt and Cot/tpl2 KO mice upon 18 h intraperitoneal injection of DAMPs (1.7 mg) are shown. Graphs represent the mean ± SD of one experiment performed in triplicate. Similar data were obtained in another three experiments.

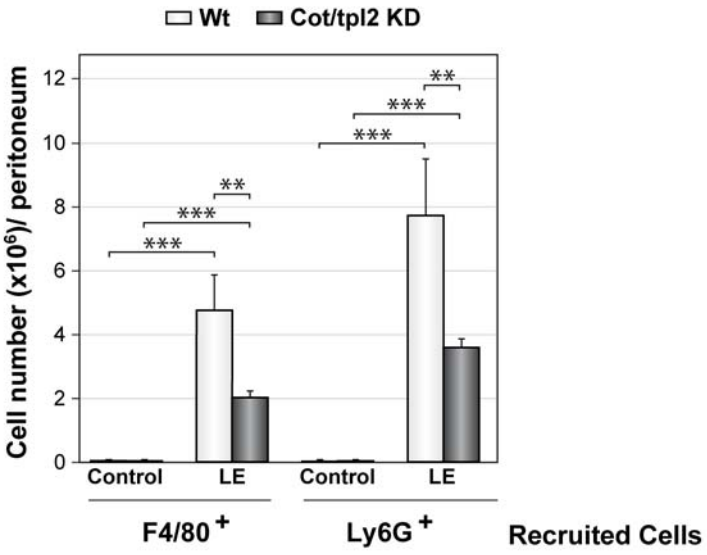


Figure S9. Leukocyte recruitment following liver extract-induced peritonitis in Wt and Cot/tpl2 KD mice**.** Animals were i.p. injected with Wt liver extract (LE, 32 mg) or with PBS (Control) and 18 h later peritoneal cells were subjected to flow cytometry analysis. Graph represents the mean ± SD of 3 independent experiments performed in triplicate.

Capítulo III

Cot/tpl-2 participates in the activation of macrophage by Adiponectin

Sanz-García, C.; Nagy, L.E.; Lasunción, M.A.; Fernández, M.; Alemany, S.

(En Revisión—Journal of Leukocyte Biology)

La Adiponectina (APN) es una hormona secretada por el tejido adiposo que sensibiliza la acción de la insulina. También hay estudios que demuestran que regula el fenotipo de macrófagos, aunque el mecanismo de acción es desconocido y su acción sobre los macrófagos confusa. En este trabajo demostramos que APN señaliza en macrófagos, pero no en hepatocitos, a través de Cot/tpl-2, siendo Cot/tpl-2 un mediador importante en la respuesta pro-inflamatoria generada tras la estimulación de macrófagos con esta hormona.

La estimulación de la línea celular de macrófagos RAW 264.7 con APN incrementa la fosforilación de IKKβ, p105-NFκB, MKK1/2, Erk1/2, JNK, p38α y Akt y promueve la degradación de IκBα. La activación se produce a concentraciones fisiológicas de APN ya que la EC50 es de 6.02 μg/ml y la concentración de APN en sangre varía desde 3-30 μg/ml. Además la adición de un inhibidor de Cot/tpl-2 bloquea la activación de Erk1/2 en respuesta a APN. En macrófagos derivados de medula ósea de ratones Wt pero no en los macrófagos Cot/tpl-2 KO se produce una fosforilación de Erk1/2 tras la estimulación con APN. Estos datos indican que Cot/tpl-2 media la activación de Erk1/2 tras la activación con APN.

AdipoR1 y AdipoR2 son los dos receptores principales por los que la APN señaliza en el hígado. Con el objetivo de estudiar la implicación de estos receptores en la activación de Erk1/2 mediada por Cot/Tpl-2 en respuesta a APN, transfectamos células RAW con siRNAs específicos de cada uno de los receptores por separado o conjuntamente. Los resultados, mostraron un aumento en la fosforilación de Erk1/2 similar al observado en células control o en células transfectadas con un siRNA control, indicando que la señalización intracelular de APN en macrófagos no está mediada por ninguno de estos dos receptores. Además la estimulación de hepatocitos Wt y Cot/tpl-2 KO, que expresan AdipoR1 y AdipoR2, reflejan unos niveles deP-Erk1/2 muy parecidos. Por otro lado, tras la estimulación con APN de macrófagos derivados de médula ósea Wt o deficientes para los receptores TLR2 y TLR4, se observan unos niveles de activación de Erk1/2 muy similares en tiempo e intensidad. Todos estos datos indican que APN no señaliza en macrófagos por ninguno de los dos TLRs que se expresan mayoritariamente en membrana plasmática ni por los receptores AdipoR1 y AdipoR2.

Posteriormente, realizamos un micro-array con RNA extraído de macrófagos Wt estimulados o no durante 18 horas con APN. Los dos grandes grupos de genes que presentan un mayor cambio de expresión están incluidos en respuesta inflamatoria y enfermedades infecciosas. Además de los 12 genes cuya expresión se incrementa un mayor nº de veces tras la estimulación, 6 de ellos están incluidos en estos grupos y más concretamente en el subgrupo de interacción citoquina-receptor. El análisis de expresión de estos 12 mRNAs se comprobó por qRT-PCR, para lo cual utilizamos RNA de macrófagos Wt y Cot/tpl-2 KO

estimulados 0, 3 y 18 horas con APN y Cot/tpl-2 regula la expresión de casi todos ellos aunque no siempre del mismo modo. Por otro lado, los 8 mRNAs cuya expresión se ve más disminuida por la estimulación con APN, se llevan a cabo independientemente de la expresión o no de Cot/tpl-2.

Dentro de todos los genes englobados en la respuesta inflamatoria tras la estimulación de macrófagos con APN, la modulación de la expresión de genes pertenecientes al grupo de interacción citoquina-receptor son los que tienen un mayor cambio. Además los datos de modulación de la expresión de estás citoquinas a nivel de mRNA coincide en general, con lo observado a nivel de su concentración en el medio extracelular de macrófagos estimulados. Curiosamente, aunque los niveles del mRNA de CCL3 son muy parecidos en macrófagos Wt y Cot/tpl-2 KO los macrófagos Wt secretan más CCL3 que los Cot/tpl-2 KO y mientras los niveles de mRNA de IL-6 son mayores en los macrófagos Cot/tpl-2 KO que en los Wt, la concentración de esta citoquina en el medio extracelular es muy similar en ambos tipos celulares. Estos datos sugieren que Cot/tpl-2 puede regular también la traducción de algunos mRNAs tras la activación de macrófagos con APN al igual que regula dicha traducción en macrófagos estimulados con LPS (ver artículo 1).

Actualmente hay controversia sobre si la APN atenúa o no las señales pro-inflamatorias generadas por la estimulación de los TLRs especialmente del TLR4. Nuestros datos indican que APN tiene un papel claramente pro-inflamatorio utilizando algunas vías de señalización intracelular que también son activadas por los TLRs, lo cual podría explicar el fenómeno de desensibilización producido por una estimulación previa con APN a la señal generada por la subsiguiente estimulación con LPS. En este contexto, en este trabajo también se demuestra que APN promueve la fagocitosis de bolas recubiertas de Zymosan un proceso llevado a cabo por los macrófagos con fenotipo pro-inflamatorio y Cot/tpl-2 participa en este proceso, mientras que la incorporación de oxLDL o acLDL no es dependiente de Cot/tpl-2 tras la estimulación de los macrófagos con APN.

El doctorando se encargó de la extracción de RNA para qRT-PCR y proteínas para western blot de todas las líneas celulares utilizadas. Realizó los ensayos de transfección con células RAW, así como los extractos para qRT-PCR y western blot. Puso a punto el sistema de tinción con Oil-Red y las inmunoflurescencias, necesario para el estudio del contenido lipídico celular y capacidad fagocítica, respectivamente. Ayudó a los análisis cuantitativos de la expresión génica, en la escritura de la publicación, y en la creación y corrección de las diferentes figuras.

Cot/tpl2 participates in the activation of macrophages by adiponectin

Carlos Sanz-Garcia[1], Laura E. Nagy[2], Miguel A. Lasunción[3], Margarita Fernandez[1], Susana Alemany[1]

[1] Instituto de Investigaciones Biomédicas Alberto Sols (CSIC-UAM), Arturo Duperier 4, 28029 Madrid, Spain. [2]Pathobiology and Gastroenterology, Cleveland Clinic, Cleveland, Ohio 44195. [3]Servicio de Bioquímica-Investigación, Hospital Universitario Ramón y Cajal, IRyCIS, Madrid, and CIBER de Fisiopatología de la Obesidad y Nutrición (CIBERobn), ISCIII, Spain.

Summary Sentence: Adiponectin is a new signal that activates Erk1/2 through the IKKβ-p105/NFκB1-Cot/tpl2 intracellular pathway in macrophages. This cassette mediates the activation of macrophages by this hormone.

Running Title: Cot/tpl2 in adiponectin signaling

Address correspondence to:

Susana Alemany

IIB.CSIC-UAM

Arturo Duperier 4, 28029 Madrid Spain

Tel: +34 91 497 54187, Fax: +34 91 585 4401

salemany@iib.uam.es

Key words: MAP kinases, M1 polarization, mircroarray, phagocytosis, foam cells, fat-derived hormone.

Total Character number: 5510; **Total Figure number**: 9; **Color Figure**: 0; **Reference number:** 83**; Abstract words:** 248**; Summary sentence words:** 25.

Abbreviations: Adiponectin, APN; bone marrow-derived macrophages, BMDM; green fluorescence protein (GFP).

While the main function of adiponectin is to enhance insulin activity, it is also involved in modulating the macrophage phenotype. Here, we demonstrate that at physiological concentrations, adiponectin activates Erk1/2 via the IKKβ-p105/NFκB1-Cot/tpl2 intracellular signal transduction cassette in macrophages. In peritoneal macrophages stimulated with APN, Cot/tpl2 influences the ability to phagocytose beads. However, Cot/tpl2 did not modulate the known capacity of adiponectin to decrease lipid content in peritoneal macrophages in response to treatment with oxidized or acetylated LDL. A microarray analysis of gene expression profiles in bone marrow-derived macrophages exposed to adiponectin revealed that adiponectin modulated the expression of about 3,300 genes; the most significantly affected biological functions were the inflammatory and the infectious disease responses. qRT-PCR analysis of Wt and Cot/tpl2 KO macrophages stimulated with adiponectin for 0, 3, and 18 h revealed that Cot/tpl2 participated in the upregulation of adiponectin target inflammatory mediators included in the cytokine-cytokine receptor interaction pathway (KEGG ID 4060). In accordance with these data, macrophages stimulated with adiponectin increased secretion of cytokines and chemokines, including IL-1β, IL-1α, TNFα, IL-10, IL-12, IL-6, and CCL2. Moreover, Cot/tpl2 also played an important role in the production of these inflammatory mediators upon stimulation of macrophages with adiponectin. It has been reported that different type of signals that stimulate TLRs, IL-1R, TNFR, FcyR and proteinase-activated receptor-1 activate Cot/tpl2. Here we demonstrate that adiponectin is a new signal that activates the IKKβ-p105/NFκB1-Cot/tpl2-MKK1/2-Erk1/2 axis in macrophages. Furthermore, this signaling cassette modulates the biological functions triggered by adiponectin in macrophages.

Introduction

Macrophages are characterized by their diversity and plasticity, having the capacity to acquire a variety of different functional phenotypes in response to the extracellular signals in their milieu (reviewed in [1, 2]). Activation of the toll-like receptors (TLRs) and IFNγ receptors promotes the production of classically activated M1-type macrophages with a pro-inflammatory profile, which play an essential role in host defense. By contrast, IL-4 receptor stimulation produces alternatively activated macrophages specialized in wound healing that contribute to the production of the extracellular matrix. Generation of a third macrophage type, termed regulatory macrophages, involves a variety of extracellular signals, including prostaglandins, IL-10 and adenosine-mediated signaling (reviewed in [1, 3, 4]).

Receptors of the TLR family are activated by different pathogen- and damage-associated molecular patterns, and they sense infection and tissue damage [5, 6]. Together with members of the IL-1R family, these receptors form the TLR/IL-1R superfamily, which transmit signals through their intracellular Toll/interleukin-1 receptor (TIR) domain. Different TLR/IL-1R receptors employ distinct adaptors to confer specificity to individual TLR/IL-1R-mediated signaling pathways. Accordingly, the MYD88 or TRIF adaptors mediate activation of the two main signaling pathways coupled to receptors of the TLR/IL-1R superfamily [7]. In general, activated MYD88 transduces signals via the E3 ubiquitin protein ligase TRAF6, signaling that is crucial to trigger downstream events like the activation of the TAK1 complex constituted by TAK1 (MAP3K7) and two TAK1 binding proteins TAB2 and TAB3 [8, 9]. TAK1 can also be activated by other upstream effectors and it plays a key role in the activation of downstream events involved in both innate and adaptive immune responses (reviewed in [10, 11]). In macrophages, TAK1 activates MKK7 and MKK3/6, resulting in the

subsequent activation of JNK and p38α. TAK1 also activates the classical IKK complex made up of IKKα, IKKβ and NEMO [7, 12, 13]. However, although TAK1 has been reported to function as an essential and positive regulator in the proinflammatory signaling cascade, recent reports provide evidence for cell type-specific functions of TAK1. For example, in neutrophils stimulated with LPS, TAK1 provides negatively regulates proinflammatory responses [14] and, in B cells, TAB2 and TAB3, but not TAK1, are critical for the activation of MAPKs [15, 16].

When macrophages are stimulated with different TLR agonists, activated IKKβ phosphorylates IκBα and p105/NFκB1, targeting IκBα for degradation and p105/NFκB1 for partial proteolysis. In resting cells, Cot/tpl2 (MAP3K8) forms a stable and inactive complex with p105/NFκB1 and ABIN2 (A20-binding inhibitor of NF-κB2), protecting Cot/tpl2 from degradation. However, the partial proteolysis of p105/NFκB1 to p50 NF-κB releases Cot/tpl2 from the complex (reviewed in [17, 18]), allowing it to activate MKK1/2 and consequently, Erk1/2 [19-21]. Cot/tpl2 is then rapidly degraded via the proteasome pathway [22, 23]. Cot/tpl2 is the only MAP3K that activates the Erk1/2 pathway under these conditions and it fulfills a unique role in the production of chemokines and cytokines upon macrophage activation (reviewed in [17, 18]). Indeed, Cot/tpl2 plays a central role in inflammatory processes that cannot be substituted by any other protein [17, 24, 25]. Moreover, Cot/tpl2 is also activated by FcγR signals as well as by stimulation of proteinase-activated receptor-1, a thrombin-activated G protein-coupled receptor, although the mechanism remains unknown [26, 27].

Adiponectin (APN), via binding to AdipoR1 and AdipoR2, sensitizes cells to insulin [28] and also modulates inflammatory processes (reviewed in [29, 30]). APN is secreted almost exclusively from adipose tissue in an inverse relation to the body mass index

[31]. It self-associates to form a homotrimer, which can in turn self-associate to form hexamers, dodecamers, etc., producing complexes of over 400 kDa. APN also exists in a globular form that is generated by proteolytic cleavage of the monomer [28, 31]. APN represents 0.01-0.05% of total serum protein (3-30 μg/ml) and its plasma levels decrease in a number of metabolic disorders, including obesity, insulin resistance, type 2 diabetes and coronary artery disease (reviewed in [29, 30]). In liver and muscle, APN signals via two receptors, AdipoR1 and AdipoR2, which are 67% homologous at the protein level [32]. AdipoR1 and AdipoR2 are members of a newly described class of heptahelix receptors which use the APPL1 as their main adaptor. APPL1 mediates the phosphorylation of p38α and AMPK [33, 34], two kinases known to play an important role in APN signaling [32, 35, 36]. However, more recent data also suggests that APN represses gluconeogenic gene expression in mouse liver independently of AMPK signaling [37] . It has been also reported that Erk1/2 phosphorylation by APN occurs in vascular and HEK293 cells [38]. In HEK293 cells, a 60-75% simultaneous reduction of both AdipoR1 and AdipoR2 mRNAs reduced Erk1/2 phosphorylation by 50%. The addition of different Src inhibitors also blocks the induction of Erk1/2 phosphorylation by APN [38].

APN also signals through calreticulin on the macrophage cell surface promoting the cleareance of early apoptotic cells [39] and T-cadherin also binds hexameric and high molecular weight complexes of APN in endothelial and smooth muscle cells [40, 41]. However, T-cadherin is a glycosylphosphatidylinositol anchored protein, and therefore, has to interact with a yet not identified transmembrane receptor protein for signal transduction [40, 41]. This interaction of APN with T-cadherin plays a critical role in APN-mediated cardioprotection in mice [31, 42].

Given its capacity to attenuate the inflammatory responses induced by LPS, APN has been ascribed an anti-inflammatory role [43, 44]. However, increased levels of APN are found in inflamed tissues, such as the synovium of patients with rheumatoid arthritis, and some recent findings suggest that APN acts as an inducer of proinflammatory factors [45-50].

Here, we show that APN stimulates Erk1/2 phosphorylation in macrophages by activating the IKKβ-p105/NFκB1-Cot/tpl2 intracellular signaling cassette and the activation of this intracellular signaling axis by APN plays a crucial role in the polarization of macrophages to proinflammatory phenotype.

Experimental Procedures

Animals and cells. C57BL/6 Wt, C57BL/6 Cot/tpl2 KO and C57BL/6 TLR2/4 KO mice were generated by crossing heterozygous mice [25] and they were used in experiments at 10-12 weeks of age. All animals were handled in accordance with institutional guidelines for the care and use of laboratory animals in research and the relevant European Council Directive (2010/63/EU). RAW264.7 macrophage-like cells were cultured as described previously [19] and bone marrow-derived macrophages (BMDM) or resident peritoneal macrophages were generated as described previously [51]. The human THP-1 monocytic cell line was maintained as indicated for RAW264.7 cells and differentiated as indicated previously (falta refrencia-monocyte to macrophage differentiation 2012). Immortalized Wt and Cot/tpl2 KO hepatocyte cell lines were generated from pools of 4-6 livers from Wt and Cot/tpl2 KO neonates (3.5-4-day-old) mice, which were submitted to collagenase dispersion and placed in primary culture. Subsequently, viral Bosc-23 packaging cells were transfected at 70% confluence with 3 µg/6 cm-dish of the puromycin-resistance retroviral vector pBabe encoding SV40 Large T antigen (provided by J. de Caprio, Dana Farber Cancer Institute, Boston, MA). Neonatal hepatocytes (60% confluence) were infected with polybrene (4 µg/ml) supplemented virus and maintained in culture medium for 72 h before selection with puromycin (1 µg/ml) for 10–15 days. Pools of infected cells rather than individual clones were selected to avoid potential clone-to-clone variations. Then, these pools of immortalized neonatal hepatocytes were further cultured with arginine-free medium supplemented with 10% FBS for at least 2 weeks to avoid growth of non-parenchymal cells. Cells were grown in DMEM-Hepes containing 10% FBS, Glutamine 2mM and 100 U/ml Pen/Strep.

Stimuli and inhibitors. Recombinant full length APN or Control-APN were obtained, from the supernatant of 293-HEK cells transfected either with pFMI-GFP-Adiponectin or with pFM1-GFP, as described previously [52]. Briefly, 293-T cells were transfected with the pFMI just containing the cDNA of green fluorescence protein (GFP) or with the pFM1 vector containing the cDNA of GFP together with the full length cDNA encoding APN. These plasmids (a generous gift from PE Scherer, Dallas, USA) were transfected into the cells using Lipofectamine 2000 (Invitrogen) and GFP-APN- as well as GFP-cells strongly expressing GFP were selected by 4 consecutive rounds of FACS-Sorter. Cells were propagated in growth medium (DMEM containing 10% FBS, 100 U/ml Pen/Strep and 0.1 g/L vitamin C). To collect APN from the medium of the GFP-APN-cells, the growth medium was removed, the cells were washed 3 times with PBS and serum-free DMEM containing 0.1 g/L vitamin C. Cells were allowed to secrete APN in this medium for 48 h and the medium was then collected, removing all cellular debris by centrifugation at 3000 *g* x 10 min and filtration through a 0.45 μm filter. Protein was precipitated from the medium with 40% w/v ammonium sulfate on ice, stirring for 4 h, and the precipitate was pelleted at 3000 *g* x 1 h and resuspended in 20 mM Hepes [pH 8.0] plus 50 mM NaCl (low-salt column buffer). This mixture was passed over a 5 ml EconoPac High Q anion exchange cartridge (Bio-Rad) and washed with 100 ml of low-salt column buffer. APN was then eluted from the column using an increasing salt gradient from 50 to 400 mM NaCl, starting with low-salt column buffer. APN was subsequently concentrated using a 10 kDa NMWL-Centricon (Millipore Corporation) and washed several times with 50 mM NaCl for use in all experiments. To obtain Control-APN the same procedure as the one described for GFP-APN-cells, but with GFP-cells, was performed. Acetylated (ac) low density lipoproteins (LDL) or oxidized (ox) LDL were obtained by isolation of human LDL and chemical

modification of LDL were performed as described [53, 54]. Briefly, acLDL was prepared from LDL by the addition of acetic anhydride and oxLDL was obtained by incubating LDL with $CuSO_4$. Modified lipoproteins were exhaustively dialyzed against buffered saline and filtered through 0.4-μm filters before use. Oxidation and acetylation of LDL were checked by agarose gel electrophoresis. BMDM, resident peritoneal macrophages, RAW cells, or hepatocytes were treated with full length APN, Control-APN, or commercial recombinant full length APN produced in 293-HEK cells (R&D Systems), *LPS (Salmonella typhimurium*; L726; Sigma-Aldrich), recombinant murine IL-10 (PeproTech), anti-murine IL-10 antibody (PrepoTech), control isotype IgG (Sigma–Aldrich), zymosan-coated fluorescent BioParticles (Invitrogen), latex beads conjugated to GFP (1-μm-diameter, Sigma-Aldrich) or with acLDL or oxLDL. Cot/tpl2 inhibitor (C1) and IKKβ inhibitor (B1605906), generously provided by Sir Philip Cohen, Dundee Scotland, were added 1 h prior to stimulation to the cells.

Transfection of RAW macrophages. For siRNA knockdown experiments, RAW cells were transfected using the Amaxa Nucleofector apparatus (Lonza). Briefly, 2 x 10^6 cells were resuspended in 105 μl of nucleofector solution and they were nucleofected with 100 nM of a specific or scrambled siRNA in the Nucleofector device using the D-032 program, as described previously [55]. Validated Silencer Select siRNA predesigned sequences were purchased from Ambion/Applied Biosystems. Transfected cells (5 x 10^4) were seeded in quadruplicate in 96-well plates and cultured for 24 h after the addition of pre-warmed DMEM (37ºC). Subsequently, mRNA was extracted from the cells and the efficiency of knockdown was determined by qRT-PCR using specific primers (Supplemental Table 1) [55]. The remaining nucleofected cells were transferred to 12-well plates, pre-warmed DMEM (37ºC) was added and 24 h later, the effect of exposure to APN (12 μg/ml) was evaluated by immunoblotting.

Immunofluorescence. Resident peritoneal macrophages (2.5 x 10^5) were plated on glass chamber slides (Thermo Scientific) and incubated in medium supplemented with 10% FBS and antibiotics. Subsequently, the cells were stimulated with APN (12 μg/ml) and 18 h later, latex fluorescent beads in a ratio of 10 beads per cell or zymosan fluorescent beads (5 μg/ml) were added. The chamber slides were then incubated for 3 h at either 37ºC or 4ºC, and cells were washed several times with PBS, fixed for 10 min in 4% PFA (in PBS), permeabilized for 30 min with PBS containing 0.1% Triton X-100 and blocked for 30 min with 1% BSA. After 3 washes with PBS, cells were co-stained for 20 min at room temperature with DAPI (1:5,000, Molecular Probes) and phalloidin (1:500, Invitrogen) to label the nucleus and actin, respectively. Images from 3 different experiments performed in triplicate were taken using a 40x objective and the incorporation of the different types of beads was quantified in 4 randomly selected microscopy fields from each sample using NIH Image J software.

Oil-Red staining. Resident peritoneal macrophages were plated on chamber slides as described above, incubated in culture media supplemented with 0.1% FBS overnight and then incubated for 18 h in the presence or absence of APN (12 μg/ml). Subsequently, acLDL or oxLDL (40 μg/ml) were added for 9 h and after several washes with PBS, the cells were fixed for 10 min in 4% PFA (in PBS), rinsed for 3 min in 60% isopropanol to facilitate the staining of neutral lipids, washed twice with PBS and stained for 10 min in the dark with freshly prepared pre-warmed (37ºC) diluted Oil-Red solution (6:4 stock solution:water). The Oil-Red stock solution (Sigma-Aldrich) was prepared as described previously [56]. After 2 washes with PBS, cells were stained for 3 min with Carazzi's Hematoxylin solution (DC, Panreac) and washed several times before mounting. The incorporation of oxLDL and acLDL was quantified in 4 randomly

selected microscopy fields from each sample using a 20x objective and NIH Image J software (4 different experiments were performed in quadruplicate).

Immunoblotting and qRT-PCR analysis. Cell extracts were analyzed by immunoblotting, as described previously [57], probed with primary antibodies raised against the following proteins: Cot/tpl2, tubulin, Erk2, Akt, p38α and p52 JNK2 (Santa Cruz Biotechnology); P-S933 p105/NFκB1, P-T202/Y204 Erk1/2, P-T180/Y182 p38α, P-S176/S180 IKKα/β, P-S473 Akt, P-T286/P-S298 MKK1/2, IκBα, P-T705 STAT3, and P-T172 AMPK (Cell Signaling); or P-T183/Y185 p48/p52 JNK (Invitrogen), where P stands for phosphorylated amino acid residue. Secondary antibodies raised against rabbit (Cell Signaling), goat (DAKO) and mouse (Amersham Biosciences) were used to detect the primary antibodies. The NIH ImageJ program was used to quantify the immunoblots. RNA extraction and qRT-PCR analysis was performed as described previously [58], and specific IL-1α and IL-12β TAQMAN (Applied Biosystems) and SYBER primers (Sigma-Aldrich) were used (Supplemental Table 1).

DNA Microarray and data analysis. Microarray hybridization, imaging and data analysis were performed by Bioarray Diagnóstico Genético (Crevillente, Alicante, Spain) using RNA isolated from 3 different experiments. The methodology as well as the data analysis has been described in http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49332.

Differentially expressed genes were identified as those that exhibited changes of at least 2-fold and adjusted *P* values of ≤0.05 were returned from 3 different microarrays.

Statistical analysis. The data are presented as the mean ± SD and analyzed by the Student´s t-test. Values were considered statistically significant at: *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Results

Erk1/2 activation in macrophages stimulated with APN. Stimulation of TLR4 by LPS requires Cot/tpl2 to activate Erk1/2 ([17, 51]). APN stimulation of BMDM also requires Cot/tpl2, but is independent of TLR2 or TLR4 expression to trigger Erk1/2 phosphorylation (Fig. 1A), however LPS that stimulates via TLR4, does not activate Erk1/2 in TLR2 2/4 KO cells (Fig. S1A). We have previously shown that Cot/tpl2-MKK1/2-Erk1/2 signaling regulates Akt phosphorylation in residue S473 upon LPS activation of macrophages [51] and Cot/tpl2 also mediates Akt phosphorylation in macrophages stimulated with APN (Fig. 1B). One hour after APN stimulation achieves maximal Erk1/2 phosphorylation, but occurs more rapidly in BMDM upon stimulation with LPS (Fig. 1A, 1C, and S1). In contrast, conditioned media collected from cells not transfected with APN (Control-APN) was not able to induce Erk1/2 phosphorylation at any of the times tested (Fig. 1C). The anti-diabetic metabolic actions of APN in hepatocytes are mediated by the activation of two well characterized receptors, AdipoR1 and AdipoR2 [32]. The simultaneous use of specific AdipoR1 and AdipoR2 siRNAs decreased the mRNA expression for the receptors by 80% and 60%, respectively, in RAW cells (Fig. 1D). Under these conditions, the phosphorylation of Erk1/2 following APN stimulation did not diminish (Fig. 1E). APN stimulation of hepatocytes, which express both AdipoR1 and AdipoR2 [32], triggers both AMPK and p38α phosphorylation in both Wt and Cot/tpl2 KO hepatocytes (Fig. 1F), but did not provoke an increase in Erk1/2 phosphorylation as observed in macrophages (Fig. 1F). These data indicate that in hepatocytes Cot/tpl2 does not increases Erk1/2 phosphorylation upon activation with APN.

The IKKβ-p105/NFκB1-Cot/tpl2-MKK1/2 intracellular axis mediates Erk1/2 activation by APN in macrophages. In macrophages, Cot/tpl2-mediated Erk1/2 activation is dependent on prior phosphorylation of IKKβ, which subsequently phosphorylates p105/NFκB1. This latter phosphorylation event released Cot/tpl2 from its inactive complex with p105/NFκB1, allowing it to trigger Erk1/2 phosphorylation via MKK1/2, prior to being rapidly degraded [17, 18, 58]. The phosphorylation of Erk1/2, but not the one of p105/NFκB1, is reduced in RAW cells following the stimulation with commercial APN or LPS and in the presence of the Cot/tpl2 inhibitor, C1 (Fig. 2A and S1B). Besides, phosphorylation of both p105/NFκB1 and Erk1/2 correlated with the partial degradation of Cot/tpl2 (Fig. 2B and Fig. S1C). Stimulation of RAW as well as THP-1 macrophages with different concentrations of APN revealed that APN activates Erk1/2 with an EC50 of respectively 6.02 and 6.46 μg/ml (Fig. 2C, S1D), being the physiological plasma concentration of adiponectin in the range of 3-30 μg/ml [29, 30]. APN stimulation of RAW cells, does not only increases MKK1/2 and Erk1/2 phosphorylation but also activates JNK, and p38α (Fig. 2D). Interestingly, the ratios of Erk1/2, JNK1/2 and p38α phosphorylation are comparable when RAW cells are stimulated with APN for 60 min or with LPS for 30 min (Fig. 2D and Fig. S1E). APN also induces the phosphorylation of the upstream activator of p105/NFκB1, IKKβ, as well as IκBα degradation (Fig. 2D). Furthermore, and as described previously for LPS (Fig. S1F and [17, 18]), IKKβ activity was required for APN to activate Erk1/2 in macrophages (Fig. 2E).

Differential gene expression in APN-treated Wt and Cot/tpl2 KO macrophages. To better understand the changes produced in macrophages following APN stimulation and the role of the Cot/tpl2-MKK1/2-Erk1/2 axis in this process, we used the Affymetrix genechip system to perform an analysis of the differentially expressed genes in Wt

macrophages following stimulation with APN. An Ingenuity gene array analysis of Wt macrophages incubated in the presence or absence of APN for 18 h revealed that the two most significantly affected biological functions were the inflammatory response and infectious disease (Table 1), indeed both share a high number of common genes. The total number of genes that showed a modulation of 2-fold or greater in macrophages by the 18 h stimulation of APN was 3,233 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE49332) and of the top 12 upregulated genes 6 were members of the cytokine-cytokine receptor interaction network (Table S2). These results were confirmed by qRT-PCR analysis (Fig. 3). Furthermore, the mRNA expression analysis of these 12 upregulated genes in Wt and Cot/tpl2 KO BMDM stimulated with APN for 0, 3, and 18 h revealed that the expression of almost all these genes was modulated by Cot/tpl2, albeit not always in the same manner. In macrophages stimulated with APN, Cot/tpl2 played an important role for the expression of CXCL3, IL-1β and SAA2 (a protein involved in foam cell formation [60, 61]). Moreover, Cot/tpl2 positively modulated the expression of the HSPA1a chaperone, the TNF family member Tnfsf9, and the Adora2b adenosine receptor, the stimulation of which promotes alternatively activated macrophages [62]. Cot/tpl2 clearly represses the expression of CCR7 at 18 h. In addition, Cot/pl2 also reduces the expression of COX-2, IL-6, DUSP2, and CXCL1 mRNA in macrophages treated with APN for 3 h. However, after 18 h stimulation with APN, these mRNAs were similarly or more strongly expressed in Cot/tpl2 KO cells than in the corresponding Wt controls. In contrast, the induction of SERPINF1 mRNA by APN was independent of Cot/tpl2 expression (Fig. 3). APN also provoked the down-regulation of gene expression in Wt macrophages and the 8 most strongly down-regulated genes in macrophages treated with APN for 18 h are listed in Table S3.

However, in contrast to its role in gene upregulation in macrophages in response to APN, Cot/tpl2 did not regulate the expression of any of these 8 top downregulated genes (Fig. 4).

Our analysis using the Kyoto Encyclopedia of Genes and Genomes program indicated that the cytokine-cytokine receptor interaction pathway (KEGG ID 4060) was the most upregulated pathway in Wt macrophages following APN stimulation (P = 1.26 x 10^{-10}, Odds Ratio = 3.28). APN modulated the expression of 17% of the genes involved in this pathway and significantly, some of the cytokines that form part of this pathway are also included in the list of the 12 most upregulated genes in APN-treated macrophages (Table S2 and Fig. 3). Other representative genes included in this pathway were further analyzed by qRT-PCR. Increased IL-1α, TNFα, CXCL2, and IL-10 mRNA expression was mainly detected in Wt macrophages after 3 h stimulation, whereas the induction of CXCL9, IL-19, IL-12β, and IL-27 mRNA expression was more prominent after 18 h (Fig. 5). Cot/tpl2 deficiency drastically reduced the expression of IL-1α, TNFα, CXCL2, IL-19, and IL-10 mRNAs, whereas IL-12β and IL-27 mRNA levels were augmented in APN-stimulated Cot/tpl2 KO macrophages with respect to their Wt counterparts. However, Cot/tpl2 did not participate in the APN induction of CXCL9 and CCL3 mRNA in macrophages (Fig. 5).

Cot/tpl2 controls the production of cytokines and chemokines in macrophages following APN stimulation. To determine whether the changes in expression of the mRNAs encoding these inflammatory mediators correlated with changes in their secretion by Wt and Cot/tpl2 KO macrophages stimulated with APN, we measured extracellular levels of cytokines and chemokines after 18 h of APN stimulation and indeed APN stimulation increased the production of the chemokines and cytokines

tested (Fig. 6). In agreement with our qRT-PCR results, lack of Cot/tpl2 increased the secretion of IL-12 and CCL2 but diminished the release of TNFα, IL-1α, IL-1β, and IL-10 into the extracellular media by macrophages stimulated for 18 h with APN (Fig. 6). Furthermore, IL-6 mRNA levels were decreased in Wt macrophages respect to the levels observed in Cot/tpl2 KO cells upon APN stimulation similar IL-6 secretion was observed in both types of cells and whereas Cot/tpl2 deficiency did not affect total CCL3 mRNA, less of this cytokine was secreted in Cot/tpl2 KO macrophages compared to their Wt counterparts upon APN stimulation. Taken together, these data indicate that Cot/tpl2 controls the production of inflammatory mediators by macrophages stimulated with APN.

IL-10 partially represses COX-2, IL-6, and IL-12 mRNA levels in Wt macrophages stimulated with APN. The secretion of IL-10 upon stimulation of macrophages with LPS feedback represses the induction of COX-2 [63]. IL-10 also represses the production of IL-6 and IL-12 [53, 64, 65]. Thus, we next explored the possibility that increased IL-10 production of Wt macrophages upon stimulation with APN, compared to their Cot/tpl2 KO counterparts (Fig.6), could repress the induction of COX-2, IL-6, and IL-12β mRNA levels in these cells (Figs. 3, 5). The existence of an IL-10 autocrine feedback loop was assessed by stimulating Wt and Cot/tpl2 KO macrophages with APN in the presence and absence of an anti-IL-10 antibody. Phosphorylation of STAT3, the main IL-10 intracellular transducer [53, 64], was used as a measure of IL-10 activity. Wt macrophages, but not Cot/tpl2 KO macrophages, showed an increase in STAT3 phosphorylation, upon APN stimulation. This increase in STAT3 phosphorylation was blocked in the presence of an anti-IL-10 antibody (Fig. 7A). When Wt macrophages were stimulated with APN, inclusion of the anti-IL-10 antibody, increased the expression of COX-2, IL-12β, and IL-6 mRNA (Fig. 7B); however, this increase

reduced compared to the induction observed in Cot/tpl2 KO macrophages upon stimulation with APN. These data indicate Cot/tpl2 represses the expression of COX-2, IL-6, and IL-12β mRNA levels only partially via IL-10 in macrophages stimulated with APN.

Cot/tpl2 mediates APN-induced phagocytosis in macrophages. Phagocytosis is part of the inflammatory response. Contradictory results have been reported in relation to APN effects on macrophage phagocytosis [39, 66]. The phagocytic capacity of Wt and Cot/tpl2 KO resident peritoneal macrophages following 18 h APN stimulation was tested by the addition of fluorescence beads. Experiments performed at 37ºC indicated that Wt macrophages stimulated with APN took up these particles better than their Cot/tpl2 KO counterparts. Control experiments were performed also performed at 4ºC (Fig. 8A). Similar results were obtained when Wt and Cot/tpl2 KO resident peritoneal macrophages were subjected to phagocytose with fluorescent beads coated with zymosan (Fig. 8B), a PRR-ligand [6]. These findings indicated in macrophages, Cot/tpl2 participates in the increased phagocytosis induced by APN.

Cot/tpl2 does not influence the reduction of lipids content in macrophages upon APN stimulation. Another aspect in which APN has been specifically involved is in the reduction of lipid content in macrophages [67, 68]. To investigate the possible role of Cot/tpl2 in the capacity of APN to attenuate lipid content in foam cells, Wt and Cot/tpl2 KO resident peritoneal macrophages incubated in the presence or absence of APN were subsequently treated with oxLDL or acLDL. While APN decreased lipid in both Wt and Cot/tpl2 cells, the absence or presence of Cot/tpl2 did not interact with the action of APN (Fig. 9). These data indicate that the activation of Cot/tpl2 by APN is not involved in its ability to attenuate foam cell formation.

Discussion

The liver is the main target of APN. This hormone enhances the action of insulin in hepatocytes [28], and in addition modulates inflammatory processes. In the present study, we show that APN activates the IKKβ-p105/NFκB1-Cot/tpl2-MKK1/2-Erk1/2 intracellular axis in macrophages. The activation of this signaling cassette by ligands that activate different receptors of the TLR/IL-1R superfamily has been studied extensively [17]. In macrophages, these receptors, with the exception of TLR3 which signals via TRIF, activate TAK1 complex, the direct upstream effector of IKKβ, via MYD88-TRAF [17, 18]. However, additional signals, such as TNFα and CD40L, are also be involved in the activation of Cot/tpl2 [69]. Upon activation, TNFR1 recruits the RIP-TRAF2-TAK1 complex [70], while some other TNFR family members like TNFR2 and CD40 directly recruit different TRAFs, and subsequently TAK1 complex, to the intracellular domain of the receptors [9]. In B cells, TAB2 and TAB3 are dispensable for TAK1 activation in B cells, but critical for the activation of Erk1/2 in response to TLR and CD40 stimulation [15, 16]. In contrast, in neutrophils stimulated with LPS, TAK1 acts as a negative regulator of proinflammatory responses [14]. In addition, TAK1 can also be activated by a variety of different effectors (reviewed in [10, 11]). Cot/tpl2 is also activated by other type of receptors that are not included in the TLR/IL1R or TNFR receptor families. FcγR intracellular signaling, as well as stimulation of proteinase-activated receptor-1, a thrombin-activated G protein-coupled receptor, activates Cot/tpl2, but the mechanisms remain unclear [26, 27]. All these data open the possibility that other MAP3K, in addition to TAK1, could activate Cot/tpl2 [16].

APN binds at the surface of the plasma membrane to different type of proteins, including AdipoR1, AdipoR2, calreticulin and T-cadherin [32, 39-41]. In liver and muscle, APN signals via AdipoR1 and AdipoR2 [71]. Our data indicate that in hepatocytes upon APN stimulation, Cot/tpl2 does not regulate the phosphorylation state of Erk1/2. In HEK 273, a simultaneous 60-75% reduction in both AdipoR1 and AdipoR2 mRNA levels reduced in a 50% phosphorylation of Erk1/2 following APN stimulation, indicating that in HEK cells APN activates, at least partially, Erk1/2 through both AdipoR1 and AdipoR2 [38]. Here we show that in macrophages, the simultaneous 80% and 60% reduction in the mRNA levels of AdipoR1 and AdipoR2, respectively, did not affect Erk1/2 phosphorylation upon APN stimulation. In this context, it has been previously reported that in other hematopoietic cells, like bone marrow-derived dendritic cells, APN activates JNK and induces IL-12 secretion independently of AdipoR1 or AdipoR2 expression [50]. The crystal structure of a homotrimeric fragment from APN at 2.1 Å resolution reveals an unexpected homology with TNF family members [72], and its shares structural similarity with collagens VIII and X, as well as complement factor C1q, which is involved in the recognition of microbial surfaces and antibody-antigen complexes in the classical complement pathway [31]. Thus, the possibility that, in macrophages, APN modulates the IKKβ-p105/NFκB1-Cot/tpl2-MKK1/2-Erk1/2 axis independently of AdipoR1 and AdipoR2 cannot be excluded.

Here we show that Cot/tpl2 participates in the activation of macrophages by APN, it participates in the induction of inflammatory mediators and in the phagocytosis induced by APN, but not in the reduction of foam cell formation. These data indicate that not all the functions triggered by APN in macrophages are mediated by Cot/tpl2. Both anti-inflammatory as well as pro-inflammatory actions has been ascribed to APN [29, 30,

43, 44, 49, 50, 73, 74]. Our data support the view that APN stimulation of macrophages induces a proinflammatory M1 profile. This polarization of macrophages to a proinflammatory phenotype explains the subsequent desensitization of macrophages to further proinflammatory stimuli like LPS [49, 73, 74]. Indeed, the activation of the same IKKβ-p105/NFκB1-Cot/tpl2-MKK1/2-Erk1/2 axis by PAMPs, TNFα, IL-1, and APN reinforces this hypothesis. Here we show that stimulation of Wt macrophages with physiological concentrations of APN, which in serum is in the range of 3-30 μg/ml [29, 30], enhances the inflammatory response, by increasing the expression of genes involved in the cytokine-cytokine receptor interactions, in a manner that involved Cot/tpl2 participation. In macrophages stimulated with LPS, Cot/tpl2 induces alterations in the expression of mRNA encoding genes involved in inflammation, such as IFNβ, IFNγ, TNFα, IL6, IL-12, IL-10, CCL2, IL-1β, CCL5, CCL7, CCL8, CXCL1, CXCL2, CXCL3, CXCL9, CXCL10 y CXCL13 [51, 58, 75-80]; and CCL4 e IL-8 induced by IL-1 in Hela cells [21]. Moreover in dendritic cells, Cot/tpl2 deficiency decreases CpG mRNA induction of IL-1β and IL-10, and the expression of TNFα and IL-1β by poly IC [79, 80]. As we demonstrated here, the cytokines and chemokines modulated by Cot/tpl2 in APN-stimulated macrophages have overlap with those previously reported to be regulated by Cot/tpl2 [21, 51, 58, 75, 76].

It has been also previously shown that Cot/tpl2 deficiency decreases IL-10 production and decreases COX-2 expression levels in isolated macrophages stimulated with LPS, by stimulating CREB transcription factor activity [51, 75, 83]. However, here we show that upon APN stimulation of macrophages, Cot/tpl2 participates in IL-10 production and represses COX-2 mRNA expression levels, which is partially mediated by IL-10. A feedback repression of COX-2 induction in macrophages stimulated with LPS the induction via IL-10 secretion has been previously reported [63]. In this context, in APC-

/+ mice that develop large numbers of intestinal polyps the knock-down of Cot/tpl2 reduces intestinal IL-10 levels and increases the expression of Cot/tpl2 in polyps. Besides, IL-10 also represses IL-6 and IL-12 induction [53, 64, 65] and here we show that the increased mRNA levels expression of these two cytokines in Cot/tpl2 KO macrophages APN-stimulated is partially due to the decreased secretion of IL-10.

The modulation of mRNA expression of inflammatory mediators by Cot/tpl2 was correlated with changes in protein production, except for CCL3 and IL-6. While Cot/tpl2 deficiency had no effect on total CCL3 mRNA, less of this cytokine was detected in the extracellular media of Cot/tpl2 KO macrophages compared to their Wt counterparts upon stimulation with APN. Besides, Cot/tpl2 deficiency increased IL-6 mRNA levels following 18 h of APN stimulation but similar concentration of this cytokine was observed in the extracellular media of both Wt and Cot/tpl2 KO macrophages 18 h ather APN stimulation. Cot/tpl2 participates in the translation rate of some mRNAs encoding inflammatory mediators, including IL-6, in macrophages in macrophages stimulated with LPS [58]. Thus, the possibility that Cot/tpl2 is also involved in the translation of some mRNAs in macrophages following APN stimulation cannot be discounted.

In conclusion, we demonstrate that APN stimulation of macrophages triggers Erk1/2 phosphorylation via the IKKβ-p105/NFκB1-Cot/tpl2 intracellular signal transduction pathway. Furthermore, the activation of this axis plays an important role in the M1 proinflammatory program induced by APN in macrophages.

Authorship contributions

CS performance of experiments Figs 1, 3-8 **LN** conception and design of Fig 1. **MAL** prepared the OxLDL and acLDL, conception and design of the manuscript. **MF** performance of experiments Fig 1 and 2, conception and design of the manuscript. **SA** performance of experiments Fig 2, conception and design of the manuscript.

Acknowledgments

We thank Manuel Fresno, CBM CSIC-UAM, Madrid, for the C57BL/6 TLR2/4 KO mice. Cot/tpl2 inhibitor (C1) and IKKβ inhibitor (B1605906) were a gift from Sir Philip Cohen, Dundee, Scotland; pFM1-GFP, pFMI-GFP-Adiponectin from Philipp E Scherer; New York, USA, and THP-1 cells from Marina Mojena IIBM, Madrid, Spain. We thank Philip Cohen for the critical reading of the manuscript. We thank L. Molero, and MC. Moreno for the technical work. This work was supported by grants SAF2011-24481 to SA and by NIH R01 AA011975 to LEN. CSG is a recipient of FPI fellowship.

Conflict of Interest

The authors declare no financial or commercial conflict of interest.

References

1. Sica, A. and Mantovani, A. (2012) Macrophage plasticity and polarization: in vivo veritas. J Clin Invest 122, 787-95.
2. Stout, R. D. and Suttles, J. (2004) Functional plasticity of macrophages: reversible adaptation to changing microenvironments. Journal of leukocyte biology 76, 509-13.
3. Mosser, D. M. and Edwards, J. P. (2008) Exploring the full spectrum of macrophage activation. Nature reviews. Immunology 8, 958-69.
4. Murray, P. J. and Wynn, T. A. (2011) Protective and pathogenic functions of macrophage subsets. Nature reviews. Immunology 11, 723-37.
5. Chen, G. Y. and Nunez, G. (2010) Sterile inflammation: sensing and reacting to damage. Nature reviews. Immunology 10, 826-37.
6. Takeuchi, O. and Akira, S. (2010) Pattern recognition receptors and inflammation. Cell 140, 805-20.
7. O'Neill, L. A. and Bowie, A. G. (2007) The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nature reviews. Immunology 7, 353-64.
8. Kanayama, A., Seth, R. B., Sun, L., Ea, C. K., Hong, M., Shaito, A., Chiu, Y. H., Deng, L., Chen, Z. J. (2004) TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. Mol Cell 15, 535-48.
9. Hacker, H., Tseng, P. H., Karin, M. (2011) Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nature reviews. Immunology 11, 457-68.
10. Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., Akira, S. (2005) Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol 6, 1087-95.
11. Wan, Y. Y., Chi, H., Xie, M., Schneider, M. D., Flavell, R. A. (2006) The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol 7, 851-8.
12. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, J., Chen, Z. J. (2001) TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature 412, 346-51.
13. Kawai, T. and Akira, S. (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34, 637-50.
14. Ajibade, A. A., Wang, Q., Cui, J., Zou, J., Xia, X., Wang, M., Tong, Y., Hui, W., Liu, D., Su, B., Wang, H. Y., Wang, R. F. (2012) TAK1 negatively regulates NF-kappaB and p38 MAP kinase activation in Gr-1+CD11b+ neutrophils. Immunity 36, 43-54.
15. Ori, D., Kato, H., Sanjo, H., Tartey, S., Mino, T., Akira, S., Takeuchi, O. (2013) Essential roles of K63-linked polyubiquitin-binding proteins TAB2 and TAB3 in B cell activation via MAPKs. J Immunol 190, 4037-45.
16. Ajibade, A. A., Wang, H. Y., Wang, R. F. (2013) Cell type-specific function of TAK1 in innate immune signaling. Trends in immunology 34, 307-16.
17. Gantke, T., Sriskantharajah, S., Ley, S. C. (2011) Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase. Cell research 21, 131-45.
18. Vougioukalaki, M., Kanellis, D. C., Gkouskou, K., Eliopoulos, A. G. (2011) Tpl2 kinase signal transduction in inflammation and cancer. Cancer Lett 304, 80-9.
19. Caivano, M., Rodriguez, C., Cohen, P., Alemany, S. (2003) 15-Deoxy-Delta12,14-prostaglandin J2 regulates endogenous Cot MAPK kinase kinase 1 activity induced by lipopolysaccharide. J Biol Chem 278, 52124-30.
20. Banerjee, A., Gugasyan, R., McMahon, M., Gerondakis, S. (2006) Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. Proceedings of the National Academy of Sciences of the United States of America 103, 3274-9.

21. Rodriguez, C., Pozo, M., Nieto, E., Fernandez, M., Alemany, S. (2006) TRAF6 and Src kinase activity regulates Cot activation by IL-1. Cell Signal 18, 1376-85.
22. Waterfield, M. R., Zhang, M., Norman, L. P., Sun, S. C. (2003) NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. Mol Cell 11, 685-94.
23. Gandara, M. L., Lopez, P., Hernando, R., Castano, J. G., Alemany, S. (2003) The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. Molecular and cellular biology 23, 7377-90.
24. Cohen, P. (2009) Targeting protein kinases for the development of anti-inflammatory drugs. Curr Opin Cell Biol 21, 317-24.
25. Soria-Castro, I., Krzyzanowska, A., Pelaez, M. L., Regadera, J., Ferrer, G., Montoliu, L., Rodriguez-Ramos, R., Fernandez, M., Alemany, S. (2010) Cot/tpl2 (MAP3K8) mediates myeloperoxidase activity and hypernociception following peripheral inflammation. J Biol Chem 285, 33805-15.
26. Kyrmizi, I., Ioannou, M., Hatziapostolou, M., Tsichlis, P. N., Boumpas, D. T., Tassiulas, I. (2013) Tpl2 kinase regulates FcgammaR signaling and immune thrombocytopenia in mice. Journal of leukocyte biology 94, 751-7.
27. Hatziapostolou, M., Polytarchou, C., Panutsopulos, D., Covic, L., Tsichlis, P. N. (2008) Proteinase-activated receptor-1-triggered activation of tumor progression locus-2 promotes actin cytoskeleton reorganization and cell migration. Cancer research 68, 1851-61.
28. Trujillo, M. E. and Scherer, P. E. (2005) Adiponectin--journey from an adipocyte secretory protein to biomarker of the metabolic syndrome. J Intern Med 257, 167-75.
29. Gregor, M. F. and Hotamisligil, G. S. (2011) Inflammatory mechanisms in obesity. Annual review of immunology 29, 415-45.
30. Li, L. and Wu, L. L. (2012) Adiponectin and interleukin-6 in inflammation-associated disease. Vitamins and hormones 90, 375-95.
31. Swarbrick, M. M. and Havel, P. J. (2008) Physiological, pharmacological, and nutritional regulation of circulating adiponectin concentrations in humans. Metab Syndr Relat Disord 6, 87-102.
32. Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N. H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T. (2003) Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423, 762-9.
33. Yoon, M. J., Lee, G. Y., Chung, J. J., Ahn, Y. H., Hong, S. H., Kim, J. B. (2006) Adiponectin increases fatty acid oxidation in skeletal muscle cells by sequential activation of AMP-activated protein kinase, p38 mitogen-activated protein kinase, and peroxisome proliferator-activated receptor alpha. Diabetes 55, 2562-70.
34. Hosch, S. E., Olefsky, J. M., Kim, J. J. (2006) APPLied mechanics: uncovering how adiponectin modulates insulin action. Cell Metab 4, 5-6.
35. Buechler, C., Wanninger, J., Neumeier, M. (2011) Adiponectin, a key adipokine in obesity related liver diseases. World J Gastroenterol 17, 2801-11.
36. Yamauchi, T., Kamon, J., Minokoshi, Y., Ito, Y., Waki, H., Uchida, S., Yamashita, S., Noda, M., Kita, S., Ueki, K., Eto, K., Akanuma, Y., Froguel, P., Foufelle, F., Ferre, P., Carling, D., Kimura, S., Nagai, R., Kahn, B. B., Kadowaki, T. (2002) Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med 8, 1288-95.
37. Miller, R. A., Chu, Q., Le Lay, J., Scherer, P. E., Ahima, R. S., Kaestner, K. H., Foretz, M., Viollet, B., Birnbaum, M. J. (2011) Adiponectin suppresses gluconeogenic gene expression in mouse hepatocytes independent of LKB1-AMPK signaling. J Clin Invest 121, 2518-28.

38. Lee, M. H., Klein, R. L., El-Shewy, H. M., Luttrell, D. K., Luttrell, L. M. (2008) The adiponectin receptors AdipoR1 and AdipoR2 activate ERK1/2 through a Src/Ras-dependent pathway and stimulate cell growth. Biochemistry 47, 11682-92.
39. Takemura, Y., Ouchi, N., Shibata, R., Aprahamian, T., Kirber, M. T., Summer, R. S., Kihara, S., Walsh, K. (2007) Adiponectin modulates inflammatory reactions via calreticulin receptor-dependent clearance of early apoptotic bodies. J Clin Invest 117, 375-86.
40. Asada, K., Yoshiji, H., Noguchi, R., Ikenaka, Y., Kitade, M., Kaji, K., Yoshii, J., Yanase, K., Namisaki, T., Yamazaki, M., Tsujimoto, T., Akahane, T., Uemura, M., Fukui, H. (2007) Crosstalk between high-molecular-weight adiponectin and T-cadherin during liver fibrosis development in rats. Int J Mol Med 20, 725-9.
41. Hug, C., Wang, J., Ahmad, N. S., Bogan, J. S., Tsao, T. S., Lodish, H. F. (2004) T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. Proceedings of the National Academy of Sciences of the United States of America 101, 10308-13.
42. Denzel, M. S., Scimia, M. C., Zumstein, P. M., Walsh, K., Ruiz-Lozano, P., Ranscht, B. (2010) T-cadherin is critical for adiponectin-mediated cardioprotection in mice. J Clin Invest 120, 4342-52.
43. Folco, E. J., Rocha, V. Z., Lopez-Ilasaca, M., Libby, P. (2009) Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10. J Biol Chem 284, 25569-75.
44. Konter, J. M., Parker, J. L., Baez, E., Li, S. Z., Ranscht, B., Denzel, M., Little, F. F., Nakamura, K., Ouchi, N., Fine, A., Walsh, K., Summer, R. S. (2012) Adiponectin attenuates lipopolysaccharide-induced acute lung injury through suppression of endothelial cell activation. J Immunol 188, 854-63.
45. Ehling, A., Schaffler, A., Herfarth, H., Tarner, I. H., Anders, S., Distler, O., Paul, G., Distler, J., Gay, S., Scholmerich, J., Neumann, E., Muller-Ladner, U. (2006) The potential of adiponectin in driving arthritis. J Immunol 176, 4468-78.
46. Paul, G., Schaffler, A., Neumeier, M., Furst, A., Bataillle, F., Buechler, C., Muller-Ladner, U., Scholmerich, J., Rogler, G., Herfarth, H. (2006) Profiling adipocytokine secretion from creeping fat in Crohn's disease. Inflammatory bowel diseases 12, 471-7.
47. Fayad, R., Pini, M., Sennello, J. A., Cabay, R. J., Chan, L., Xu, A., Fantuzzi, G. (2007) Adiponectin deficiency protects mice from chemically induced colonic inflammation. Gastroenterology 132, 601-14.
48. Kang, E. H., Lee, Y. J., Kim, T. K., Chang, C. B., Chung, J. H., Shin, K., Lee, E. Y., Lee, E. B., Song, Y. W. (2010) Adiponectin is a potential catabolic mediator in osteoarthritis cartilage. Arthritis Res Ther 12, R231.
49. Cheng, X., Folco, E. J., Shimizu, K., Libby, P. (2012) Adiponectin induces pro-inflammatory programs in human macrophages and CD4+ T cells. J Biol Chem 287, 36896-904.
50. Jung, M. Y., Kim, H. S., Hong, H. J., Youn, B. S., Kim, T. S. (2012) Adiponectin induces dendritic cell activation via PLCgamma/JNK/NF-kappaB pathways, leading to Th1 and Th17 polarization. J Immunol 188, 2592-601.
51. Lopez-Pelaez, M., Soria-Castro, I., Bosca, L., Fernandez, M., Alemany, S. (2011) Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. Eur J Immunol 41, 1733-41.
52. Berg, A. H., Combs, T. P., Du, X., Brownlee, M., Scherer, P. E. (2001) The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. Nat Med 7, 947-53.
53. Saraiva, M. and O'Garra, A. (2010) The regulation of IL-10 production by immune cells. Nature reviews. Immunology 10, 170-81.
54. Calvo, D., Gomez-Coronado, D., Lasuncion, M. A., Vega, M. A. (1997) CLA-1 is an 85-kD plasma membrane glycoprotein that acts as a high-affinity receptor for both native

(HDL, LDL, and VLDL) and modified (OxLDL and AcLDL) lipoproteins. Arterioscler Thromb Vasc Biol 17, 2341-9.
55. Mandal, P., Pratt, B. T., Barnes, M., McMullen, M. R., Nagy, L. E. (2011) Molecular mechanism for adiponectin-dependent M2 macrophage polarization: link between the metabolic and innate immune activity of full-length adiponectin. J Biol Chem 286, 13460-9.
56. Xu, S., Huang, Y., Xie, Y., Lan, T., Le, K., Chen, J., Chen, S., Gao, S., Xu, X., Shen, X., Huang, H., Liu, P. (2010) Evaluation of foam cell formation in cultured macrophages: an improved method with Oil Red O staining and DiI-oxLDL uptake. Cytotechnology 62, 473-81.
57. Rodriguez, C., Lopez, P., Pozo, M., Duce, A. M., Lopez-Pelaez, M., Fernandez, M., Alemany, S. (2008) COX2 expression and Erk1/Erk2 activity mediate Cot-induced cell migration. Cell Signal 20, 1625-31.
58. Lopez-Pelaez, M., Fumagalli, S., Sanz, C., Herrero, C., Guerra, S., Fernandez, M., Alemany, S. (2012) Cot/tpl2-MKK1/2-Erk1/2 controls mTORC1-mediated mRNA translation in Toll-like receptor-activated macrophages. Molecular biology of the cell 23, 2982-92.
59. Deepa, S. S. and Dong, L. Q. (2009) APPL1: role in adiponectin signaling and beyond. Am J Physiol Endocrinol Metab 296, E22-36.
60. Meek, R. L., Urieli-Shoval, S., Benditt, E. P. (1994) Expression of apolipoprotein serum amyloid A mRNA in human atherosclerotic lesions and cultured vascular cells: implications for serum amyloid A function. Proceedings of the National Academy of Sciences of the United States of America 91, 3186-90.
61. Kisilevsky, R. and Tam, S. P. (2003) Macrophage cholesterol efflux and the active domains of serum amyloid A 2.1. J Lipid Res 44, 2257-69.
62. Csoka, B., Selmeczy, Z., Koscso, B., Nemeth, Z. H., Pacher, P., Murray, P. J., Kepka-Lenhart, D., Morris, S. M., Jr., Gause, W. C., Leibovich, S. J., Hasko, G. (2012) Adenosine promotes alternative macrophage activation via A2A and A2B receptors. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 26, 376-86.
63. MacKenzie, K. F., Van Den Bosch, M. W., Naqvi, S., Elcombe, S. E., McGuire, V. A., Reith, A. D., Blackshear, P. J., Dean, J. L., Arthur, J. S. (2013) MSK1 and MSK2 inhibit lipopolysaccharide-induced prostaglandin production via an interleukin-10 feedback loop. Molecular and cellular biology 33, 1456-67.
64. Murray, P. J. (2007) The JAK-STAT signaling pathway: input and output integration. J Immunol 178, 2623-9.
65. O'Garra, A., Barrat, F. J., Castro, A. G., Vicari, A., Hawrylowicz, C. (2008) Strategies for use of IL-10 or its antagonists in human disease. Immunological reviews 223, 114-31.
66. Yokota, T., Oritani, K., Takahashi, I., Ishikawa, J., Matsuyama, A., Ouchi, N., Kihara, S., Funahashi, T., Tenner, A. J., Tomiyama, Y., Matsuzawa, Y. (2000) Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. Blood 96, 1723-32.
67. Okamoto, Y., Kihara, S., Ouchi, N., Nishida, M., Arita, Y., Kumada, M., Ohashi, K., Sakai, N., Shimomura, I., Kobayashi, H., Terasaka, N., Inaba, T., Funahashi, T., Matsuzawa, Y. (2002) Adiponectin reduces atherosclerosis in apolipoprotein E-deficient mice. Circulation 106, 2767-70.
68. Tian, L., Luo, N., Klein, R. L., Chung, B. H., Garvey, W. T., Fu, Y. (2009) Adiponectin reduces lipid accumulation in macrophage foam cells. Atherosclerosis 202, 152-61.
69. Eliopoulos, A. G., Wang, C. C., Dumitru, C. D., Tsichlis, P. N. (2003) Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40. EMBO J 22, 3855-64.

70. Silke, J. and Brink, R. (2010) Regulation of TNFRSF and innate immune signalling complexes by TRAFs and cIAPs. Cell death and differentiation 17, 35-45.
71. Mao, X., Kikani, C. K., Riojas, R. A., Langlais, P., Wang, L., Ramos, F. J., Fang, Q., Christ-Roberts, C. Y., Hong, J. Y., Kim, R. Y., Liu, F., Dong, L. Q. (2006) APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nature cell biology 8, 516-23.
72. Shapiro, L. and Scherer, P. E. (1998) The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. Current biology : CB 8, 335-8.
73. Okamoto, Y., Folco, E. J., Minami, M., Wara, A. K., Feinberg, M. W., Sukhova, G. K., Colvin, R. A., Kihara, S., Funahashi, T., Luster, A. D., Libby, P. (2008) Adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis. Circ Res 102, 218-25.
74. Kredel, L. I., Batra, A., Stroh, T., Kuhl, A. A., Zeitz, M., Erben, U., Siegmund, B. (2013) Adipokines from local fat cells shape the macrophage compartment of the creeping fat in Crohn's disease. Gut 62, 852-62.
75. Risco, A., del Fresno, C., Mambol, A., Alsina-Beauchamp, D., MacKenzie, K. F., Yang, H. T., Barber, D. F., Morcelle, C., Arthur, J. S., Ley, S. C., Ardavin, C., Cuenda, A. (2012) p38gamma and p38delta kinases regulate the Toll-like receptor 4 (TLR4)-induced cytokine production by controlling ERK1/2 protein kinase pathway activation. Proceedings of the National Academy of Sciences of the United States of America 109, 11200-5.
76. Bandow, K., Kusuyama, J., Shamoto, M., Kakimoto, K., Ohnishi, T., Matsuguchi, T. (2012) LPS-induced chemokine expression in both MyD88-dependent and -independent manners is regulated by Cot/Tpl2-ERK axis in macrophages. FEBS Lett 586, 1540-6.
77. Rousseau, S., Papoutsopoulou, M., Symons, A., Cook, D., Lucocq, J. M., Prescott, A. R., O'Garra, A., Ley, S. C., Cohen, P. (2008) TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. J Cell Sci 121, 149-54.
78. Dumitru, C. D., Ceci, J. D., Tsatsanis, C., Kontoyiannis, D., Stamatakis, K., Lin, J. H., Patriotis, C., Jenkins, N. A., Copeland, N. G., Kollias, G., Tsichlis, P. N. (2000) TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell 103, 1071-83.
79. Kaiser, F., Cook, D., Papoutsopoulou, S., Rajsbaum, R., Wu, X., Yang, H. T., Grant, S., Ricciardi-Castagnoli, P., Tsichlis, P. N., Ley, S. C., O'Garra, A. (2009) TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. The Journal of experimental medicine 206, 1863-71.
80. Mielke, L. A., Elkins, K. L., Wei, L., Starr, R., Tsichlis, P. N., O'Shea, J. J., Watford, W. T. (2009) Tumor progression locus 2 (Map3k8) is critical for host defense against Listeria monocytogenes and IL-1 beta production. J Immunol 183, 7984-93.
81. Sanz-Garcia, C., Ferrer-Mayorga, G., Gonzalez-Rodriguez, A., Valverde, A. M., Martin-Duce, A., Velasco-Martin, J. P., Regadera, J., Fernandez, M., Alemany, S. (2013) Sterile inflammation in acetaminophen-induced liver injury is mediated by Cot/tpl2. J Biol Chem 288, 15342-51.
82. Serebrennikova, O. B., Tsatsanis, C., Mao, C., Gounaris, E., Ren, W., Siracusa, L. D., Eliopoulos, A. G., Khazaie, K., Tsichlis, P. N. (2012) Tpl2 ablation promotes intestinal inflammation and tumorigenesis in Apcmin mice by inhibiting IL-10 secretion and regulatory T-cell generation. Proceedings of the National Academy of Sciences of the United States of America 109, E1082-91.

83. Eliopoulos, A. G., Dumitru, C. D., Wang, C. C., Cho, J., Tsichlis, P. N. (2002) Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J 21, 4831-40.

Figure Legend

Figure 1. Erk1/2 activation in macrophages stimulated with APN. ***A,*** Wt, TLR2/4 KO and Cot/tpl2 KO BMDM were incubated with APN (12 μg/ml) and at the indicated times, the levels of P-Erk1/2 and Erk2 were measured by immunoblotting. ***B***, Wt and Cot/tpl2 KO BMDM were treated as in *A* and the levels of S473 Akt and total Akt were measured by immunoblotting. ***C***, Wt BMDM were incubated with APN (12 μg/ml in 15-30 μl), Control-APN (Cnt, 15-30 μl) or LPS (300 ng/ml) for the indicated times and treated as in *A*. ***D***, RAW cells were nucleofected with 100 nM of control (SCA), or with specific Adipo R1 (APNR1) and/or Adipo R2 (APNR2) for 24 h. Efficiency of siRNA knock-down was determined by qRT-PCR analysis. Data show the mean ± SD from 8 independent experiments expressed relative to the control. ***E***, RAW cells nucleofected with control (SCA), or with specific Adipo R1 (APNR1) and/or Adipo R2 (APNR2) for 24 h, were subsequently stimulated for 45 min with APN (12 μg/ml) and then treated as in *A*. ***F***, Wt and Cot/tpl2 KO hepatocytes were stimulated for the indicated times with APN (12 μg/ml) and the levels of P-AMPK; P-p38α, P-Erk1/2, p38α and Erk2 was determined by immunoblotting. ***A-F***. One representative experiment of the at least 3 performed is shown.

Figure 2. IKKβ-p105/NFκB1-Cot/tpl2-MKK1/2 mediates Erk1/2 activation in macrophages stimulated with APN. ***A***, RAW cells pretreated or not with 5 μM Cot/tpl2 inhibitor (C1) were stimulated for another hour with commercial APN (6 μg/ml), the levels of P-p105/NFκB1, P-Erk1/2 and Erk2 were measured by immunoblotting. ***B***, RAW cells were treated with APN (12 μg/ml) and at the marked times, the levels of the indicated proteins were measured by immunoblotting. The graph indicate the relative expression of H (high) and L (low) forms of Cot/tpl2 given the

value of 100% to the one obtained at 0 time point. ***C***, RAW cells were stimulated with different amounts of APN for 1 h. Cells were subsequently treated as in *A*. Graphs show the induction fold of P-p105/NFκB1 and P-Erk1/2 used as loading control total Erk2 levels. ***D***, RAW cells were incubated with APN (12 μg/ml) and at the marked times, the levels of the indicated proteins were measured by immunoblotting. Graphs represent the P-Erk1/2/Erk2, P-JNK/JNK2 and P-p38α/p38α values at 1 h APN or 30 min LPS stimulation given the value 1 to the control relative to 0 time point. ***E***, RAW cells incubated or not with 10 μM of the IKKβ inhibitor B1605906 (IKKβ I), were subsequently stimulated with APN (12 μg/ml) for another hour and then treated as in *A*. ***A-E***, One representative experiment of the 3 performed is shown. ***B-D,*** Graphs represent the mean ± SD of 3 independent experiments performed at least in duplicate.

Figure 3. qRT-PCR analysis of the top upregulated genes in Wt and Cot/tpl2 KO macrophages stimulated with APN. The relative mRNA expression levels of the genes indicated in the figure were analyzed in Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h and 18 h with APN (12 μg/ml). Data show the mean ± SD from 3 different BMDM preparations obtained from 2 different mice each and performed in quadruplicated.

Figure 4. qRT-PCR analysis of the top downregulated genes in Wt and Cot/tpl2 KO macrophages stimulated with APN. The relative mRNA expression levels of the genes indicated in the figure were analyzed in Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h and 18 h with APN (12 μg/ml). Data show the mean ± SD from 3 different BMDM preparations obtained from 2 different mice each and performed in quadruplicated.

Figure 5. qRT-PCR analysis of genes integrated cytokine-cytokine receptor interaction pathway in Wt and Cot/tpl2 KO macrophages stimulated with APN. The relative RNA expression levels of the genes indicated in the figure were analyzed in Wt and Cot/tpl2 KO BMDM stimulated or not for 3 h and 18 h with APN (12 μg/ml). Data show the mean ± SD from 3 different BMDM preparations obtained from 2 different mice each and performed in quadruplicated.

Figure 6. Cot/tpl2 modulates cytokine production in macrophages stimulated with APN. Wt and Cot/tpl2 KO BMDM were stimulated with APN (12 μg/ml) and the indicated cytokines and chemokines were determined in the extracellular media 18 h later using a Luminex assay. Similar results were obtained with commercial APN. The data represent the mean ± SD of 4 independent experiments performed in quadruplicated.

Figure 7. IL-10 partially represses COX-2, IL-6, and IL-12β mRNA levels in Wt macrophages stimulated with APN. ***A*** Wt and Cot/tpl2 KO BMDM were stimulated with APN (12 μg/ml) in the presence of anti-murine IL-10 antibody or of control isotype IgG (5 μg/ml) and the indicated times P-STAT3 and ERK2 expression were measured by immunoblotting. One representative experiment is shown. Graphs represent the mean ± SD of 3 independent experiments performed of P-STAT3/ Erk2 value given the value 1 to the control relative to 0 time point. ***B*** Wt BMDM were stimulated for 18 h as indicated in *A* and the relative RNA expression levels of COX-2, IL-6, and IL-12β were determined. Graph represent the induction of the indicated gene in the presence of IL-10 antibody given the value of 100 to the one obtained in the absence of anti-IL-10 antibody. Data show the mean ± SD from 3 different experiments performed in triplicated.

Figure 8. Involvement of Cot/tpl2 in the increased phagocytosis capacity of macrophages stimulated with APN. Resident Wt and Cot/tpl2 KO peritoneal macrophages were stimulated or not with APN (12 μg/ml) for18 h. Subsequently, in a ratio of 10 latex beads per cell (***A***) or zymosan A Bioparticles at 5 μg/ml (***B***), were added to the cells for 3 h at 37ºC or at 4ºC and then treated as indicated in Material and Methods. Graph represents the mean ± SE from the 3 different experiments performed in triplicate expressed relative to Wt control.

Figure 9. **Cot/tpl2 is not involved in the ability of APN to attenuate foam cell formation.** Resident Wt and Cot/tpl2 KO peritoneal macrophages were stimulated for 18 h without or with APN (12 μg/ml). Subsequently, cells were further incubated with oxLDL or acLDL (40 μg/ml) for 9 h, fixed in 4% PFA, rinsed in 60% isopropanol and stained with Oil-red work solution and hematoxyline. Graph shows the mean ± SE from the 4 different experiments performed in quadruplicate expressed relative to Wt control.

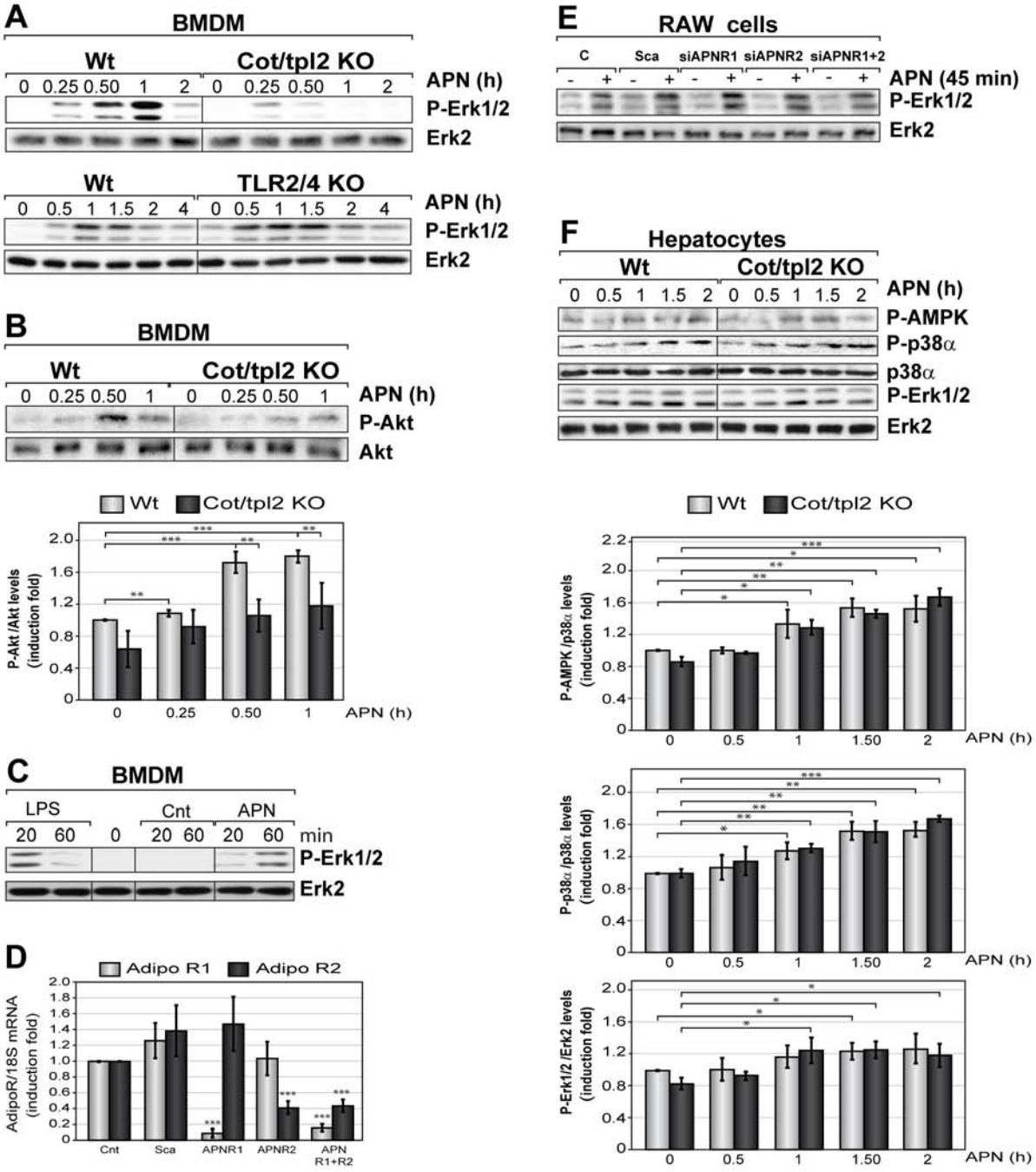

A
BMDM
Wt
Cot/tpl2 KO
0 0.25 0.50 1 2 0 0.25 0.50 1 2
APN (h)
P-Erk1/2
Erk2
Wt
TLR2/4 KO
0 0.5 1 1.5 2 4 0 0.5 1 1.5 2 4
APN (h)
P-Erk1/2
Erk2
B
BMDM
Wt
Cot/tpl2 KO
0 0.25 0.50 1 0 0.25 0.50 1
APN (h)
P-Akt
Akt
Wt
Cot/tpl2 KO
P-Akt /Akt levels (induction fold)
APN (h)
C
BMDM
LPS
Cnt
APN
20 60 0 20 60 20 60 min
P-Erk1/2
Erk2
D
Adipo R1
Adipo R2
AdipoR/18S mRNA (induction fold)
Cnt
Sca
APNR1
APNR2
APN R1+R2
E
RAW cells
C
Sca
siAPNR1
siAPNR2
siAPNR1+2
- + - + - + - + - +
APN (45 min)
P-Erk1/2
Erk2
F
Hepatocytes
Wt
Cot/tpl2 KO
0 0.5 1 1.5 2 0 0.5 1 1.5 2
APN (h)
P-AMPK
P-p38α
p38α
P-Erk1/2
Erk2
Wt
Cot/tpl2 KO
P-AMPK /p38α levels (induction fold)
P-p38α /p38α levels (induction fold)
P-Erk1/2 /Erk2 levels (induction fold)
0 0.5 1 1.50 2
APN (h)

Figure 1

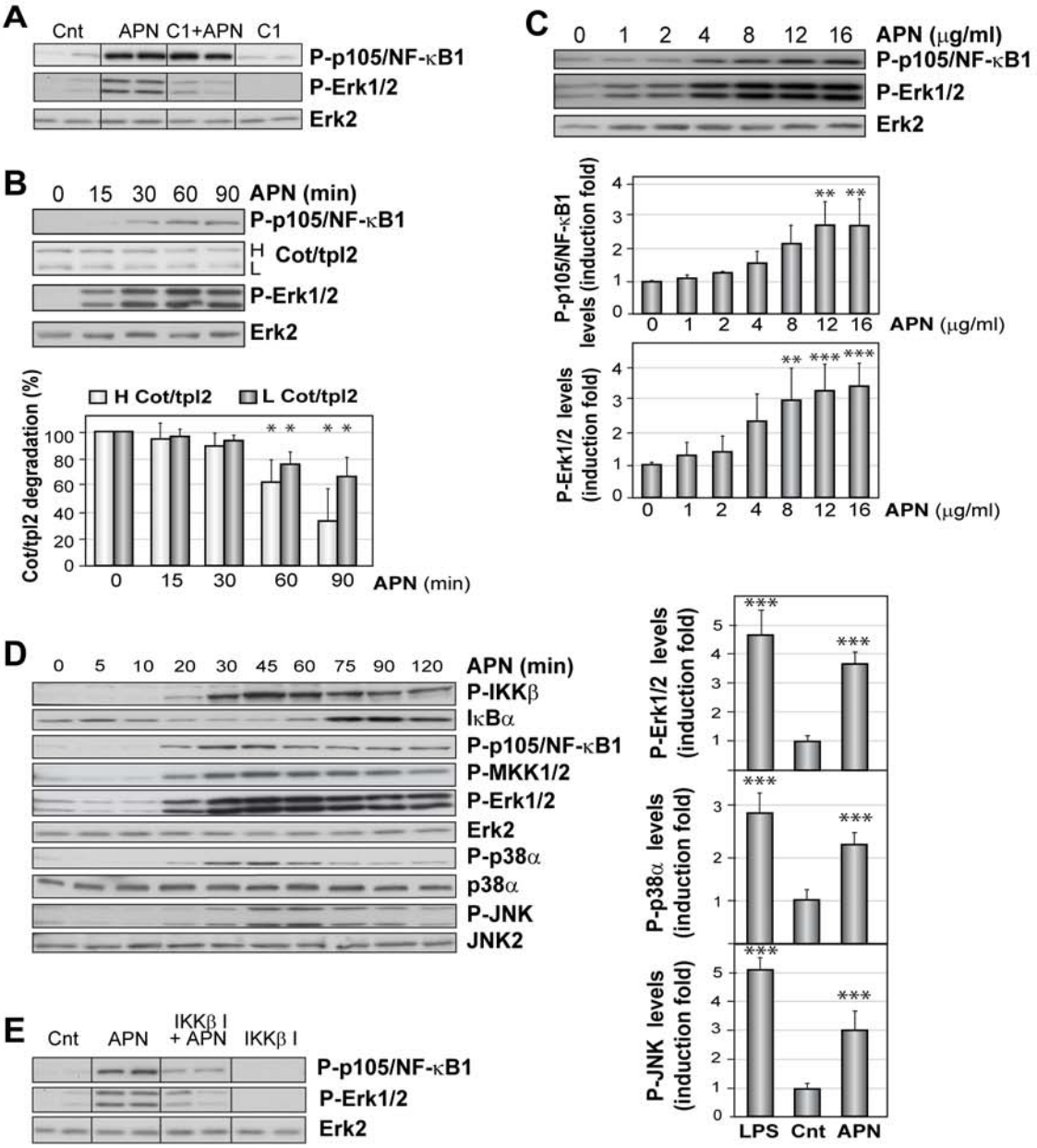

A
Cnt
APN
C1+APN
C1
P-p105/NF-κB1
P-Erk1/2
Erk2
B
0 15 30 60 90 APN (min)
P-p105/NF-κB1
H
L
Cot/tpl2
P-Erk1/2
Erk2
H Cot/tpl2
L Cot/tpl2
Cot/tpl2 degradation (%)
APN (min)
C
0 1 2 4 8 12 16 APN (μg/ml)
P-p105/NF-κB1
P-Erk1/2
Erk2
P-p105/NF-κB1 levels (induction fold)
APN (μg/ml)
P-Erk1/2 levels (induction fold)
APN (μg/ml)
D
0 5 10 20 30 45 60 75 90 120 APN (min)
P-IKKβ
IκBα
P-p105/NF-κB1
P-MKK1/2
P-Erk1/2
Erk2
P-p38α
p38α
P-JNK
JNK2
P-Erk1/2 levels (induction fold)
P-p38α levels (induction fold)
P-JNK levels (induction fold)
LPS
Cnt
APN
E
Cnt
APN
IKKβ I + APN
IKKβ I
P-p105/NF-κB1
P-Erk1/2
Erk2

Figure 2

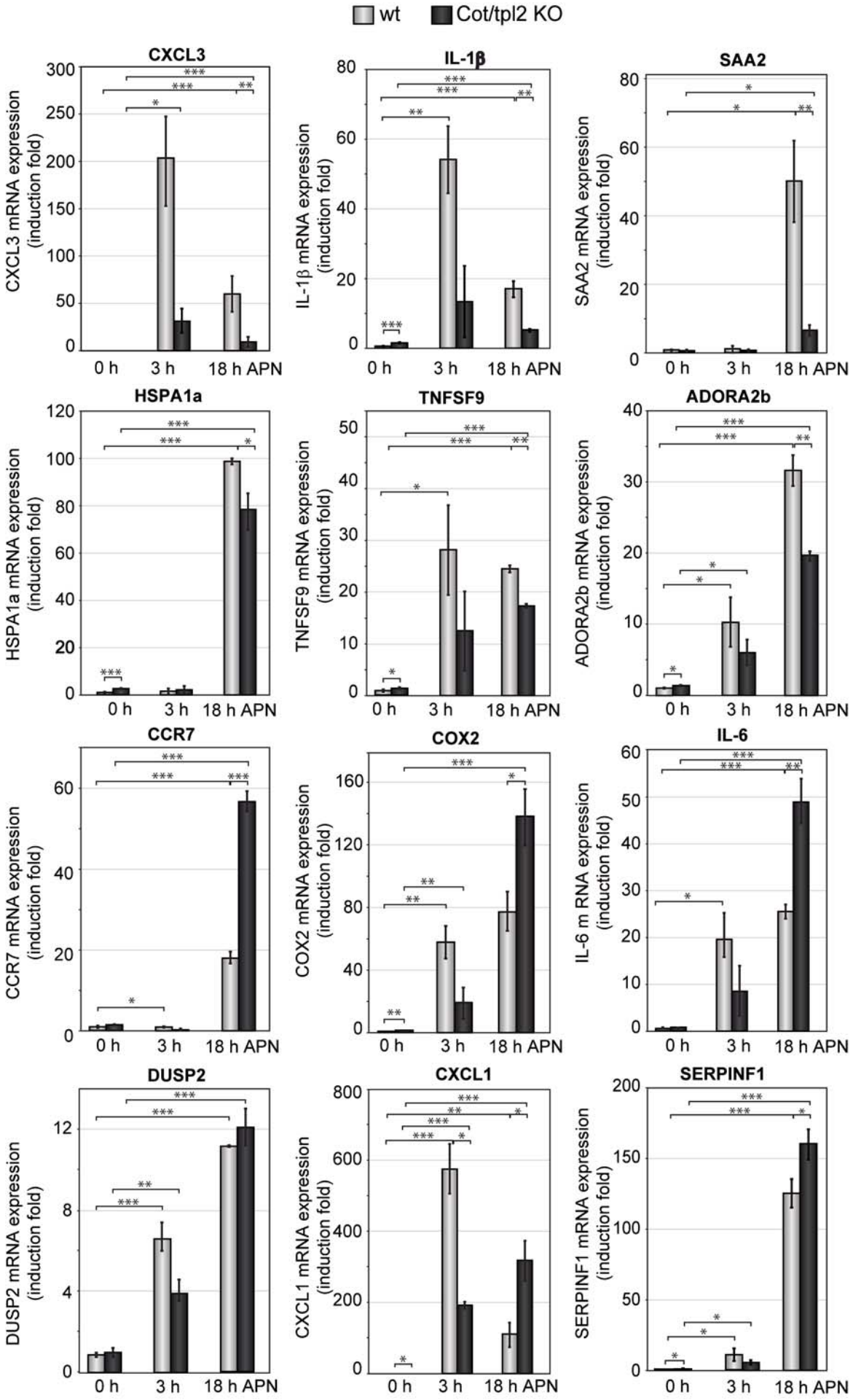

wt
Cot/tpl2 KO
CXCL3
CXCL3 mRNA expression (induction fold)
IL-1β
IL-1β mRNA expression (induction fold)
SAA2
SAA2 mRNA expression (induction fold)
HSPA1a
HSPA1a mRNA expression (induction fold)
TNFSF9
TNFSF9 mRNA expression (induction fold)
ADORA2b
ADORA2b mRNA expression (induction fold)
CCR7
CCR7 mRNA expression (induction fold)
COX2
COX2 mRNA expression (induction fold)
IL-6
IL-6 m RNA expression (induction fold)
DUSP2
DUSP2 mRNA expression (induction fold)
CXCL1
CXCL1 mRNA expression (induction fold)
SERPINF1
SERPINF1 mRNA expression (induction fold)
0 h
3 h
18 h APN

Figure 3

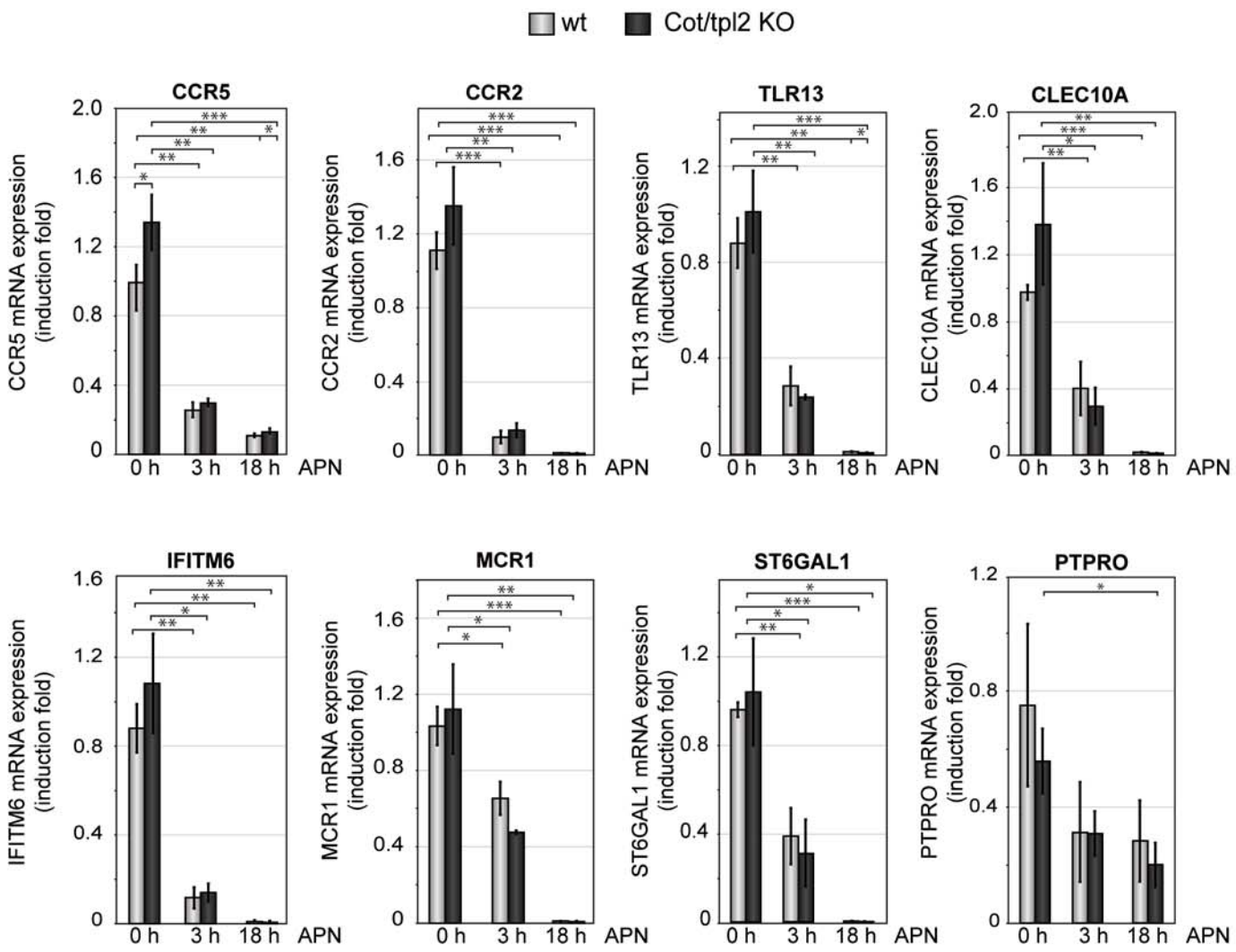

wt
Cot/tpl2 KO
CCR5
CCR2
TLR13
CLEC10A
IFITM6
MCR1
ST6GAL1
PTPRO
CCR5 mRNA expression (induction fold)
CCR2 mRNA expression (induction fold)
TLR13 mRNA expression (induction fold)
CLEC10A mRNA expression (induction fold)
IFITM6 mRNA expression (induction fold)
MCR1 mRNA expression (induction fold)
ST6GAL1 mRNA expression (induction fold)
PTPRO mRNA expression (induction fold)
0 h
3 h
18 h
APN

Figure 4

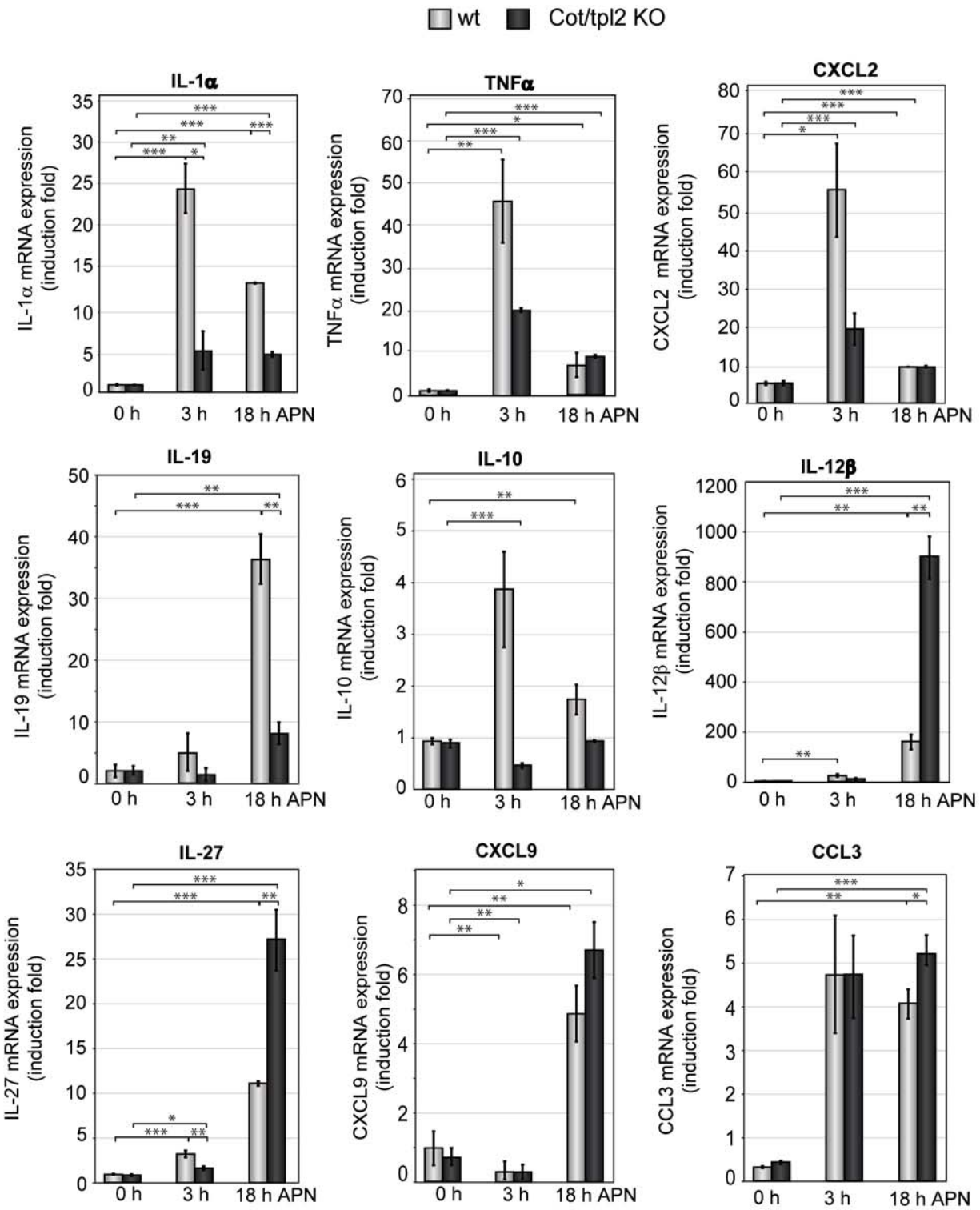

wt
Cot/tpl2 KO
IL-1α
IL-1α mRNA expression (induction fold)
TNFα
TNFα mRNA expression (induction fold)
CXCL2
CXCL2 mRNA expression (induction fold)
IL-19
IL-19 mRNA expression (induction fold)
IL-10
IL-10 mRNA expression (induction fold)
IL-12β
IL-12β mRNA expression (induction fold)
IL-27
IL-27 mRNA expression (induction fold)
CXCL9
CXCL9 mRNA expression (induction fold)
CCL3
CCL3 mRNA expression (induction fold)
0 h
3 h
18 h APN

Figure 5

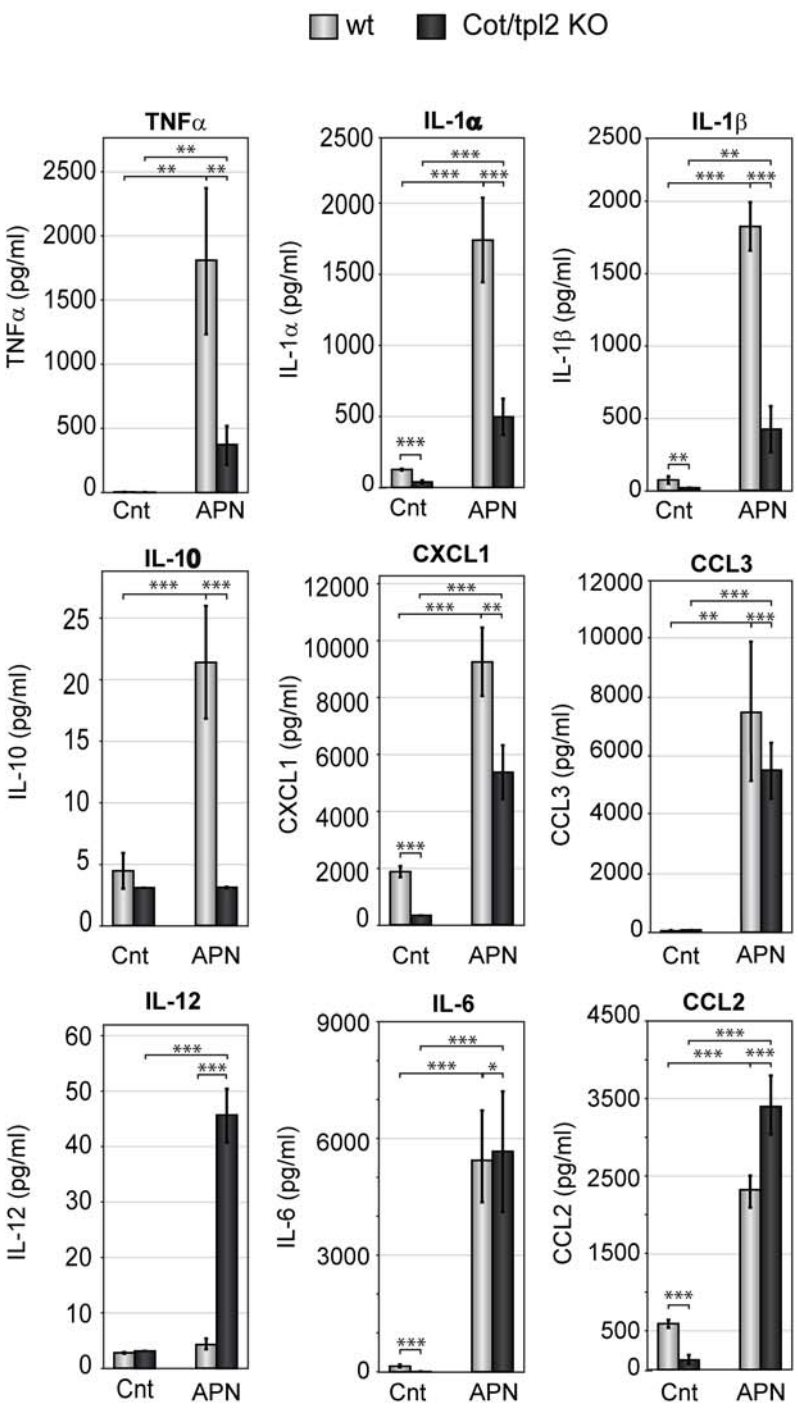

wt
Cot/tpl2 KO
TNFα
TNFα (pg/ml)
IL-1α
IL-1α (pg/ml)
IL-1β
IL-1β (pg/ml)
IL-10
IL-10 (pg/ml)
CXCL1
CXCL1 (pg/ml)
CCL3
CCL3 (pg/ml)
IL-12
IL-12 (pg/ml)
IL-6
IL-6 (pg/ml)
CCL2
CCL2 (pg/ml)
Cnt
APN

Figure 6

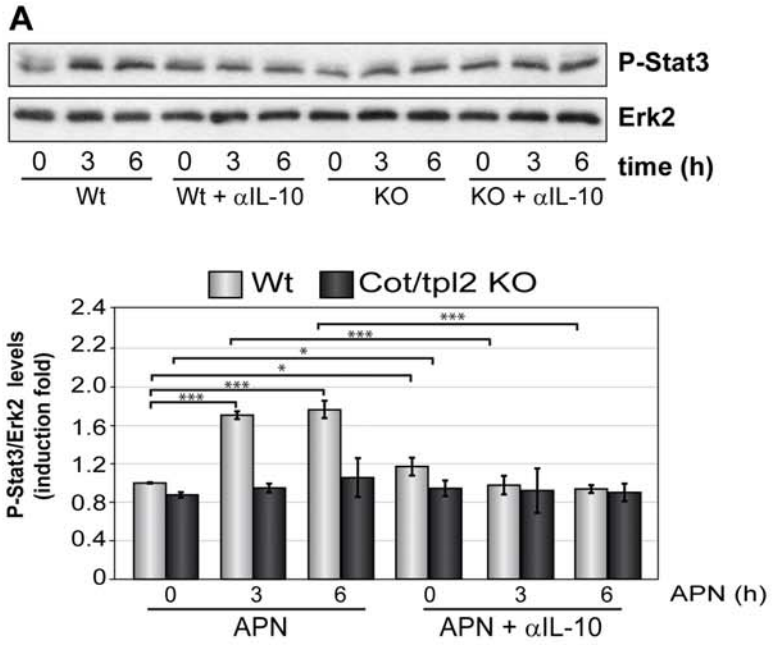

A
P-Stat3
Erk2
0 3 6 0 3 6 0 3 6 0 3 6 time (h)
Wt
Wt + αIL-10
KO
KO + αIL-10
Wt
Cot/tpl2 KO
P-Stat3/Erk2 levels (induction fold)
APN (h)
APN
APN + αIL-10
B
Wt
Wt + αIL-10
COX2 levels (induction fold %)
IL-12β levels (induction fold %)
IL-6 levels (induction fold %)

Figure 7

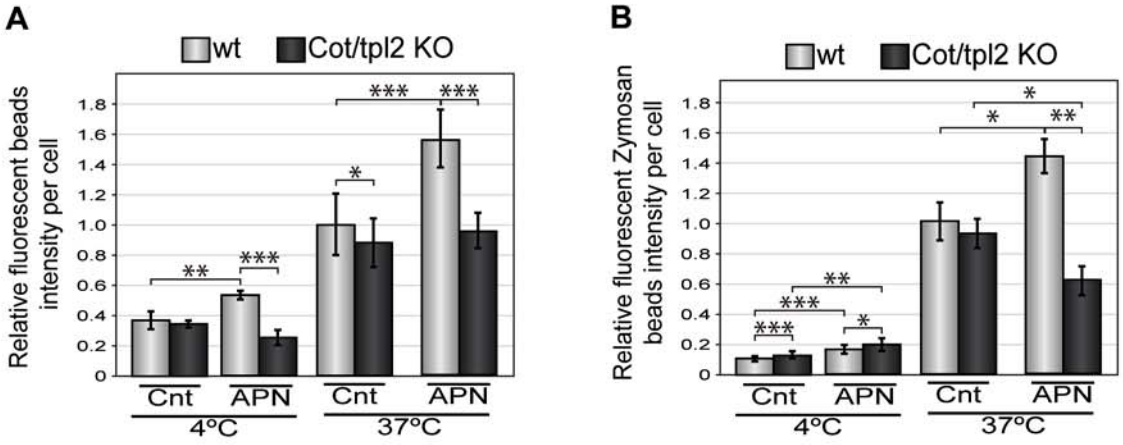

A
wt
Cot/tpl2 KO
Relative fluorescent beads intensity per cell
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
**
***
*
***
***
Cnt
APN
Cnt
APN
4°C
37°C
B
wt
Cot/tpl2 KO
Relative fluorescent Zymosan beads intensity per cell
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
***
**
*
*
**
*
Cnt
APN
Cnt
APN
4°C
37°C

Figure 8

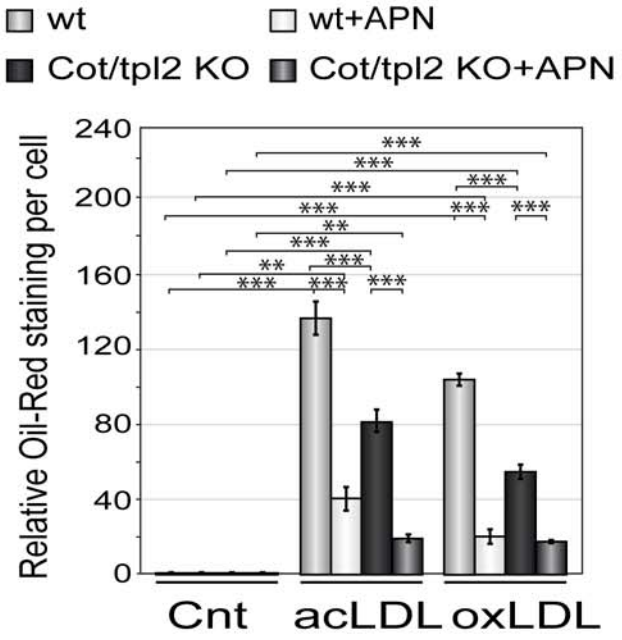

wt
wt+APN
Cot/tpl2 KO
Cot/tpl2 KO+APN
Relative Oil-Red staining per cell
240
200
160
120
80
40
0
***
**
Cnt
acLDL
oxLDL

Figure 9

TABLE 1

TOP BIO FUNCTIONS
Diseases and Disorders

Name	p value	Molecules
Inflammatory response	3.33×10^{-25} - 5.57×10^{-6}	563
Infectious disease	6.79×10^{-21} - 2.96×10^{-6}	578

Table 1 Top biological functions upregulated in APN-stimulated macrophages. An agilent genechip analysis with RNA from control and 18h APN-stimulated macrophages was performed. The top biological functions were analyzed by the ingenuity gene array analysis. Data shown are the mean of 3 different arrays performed from 3 different sets of RNA .

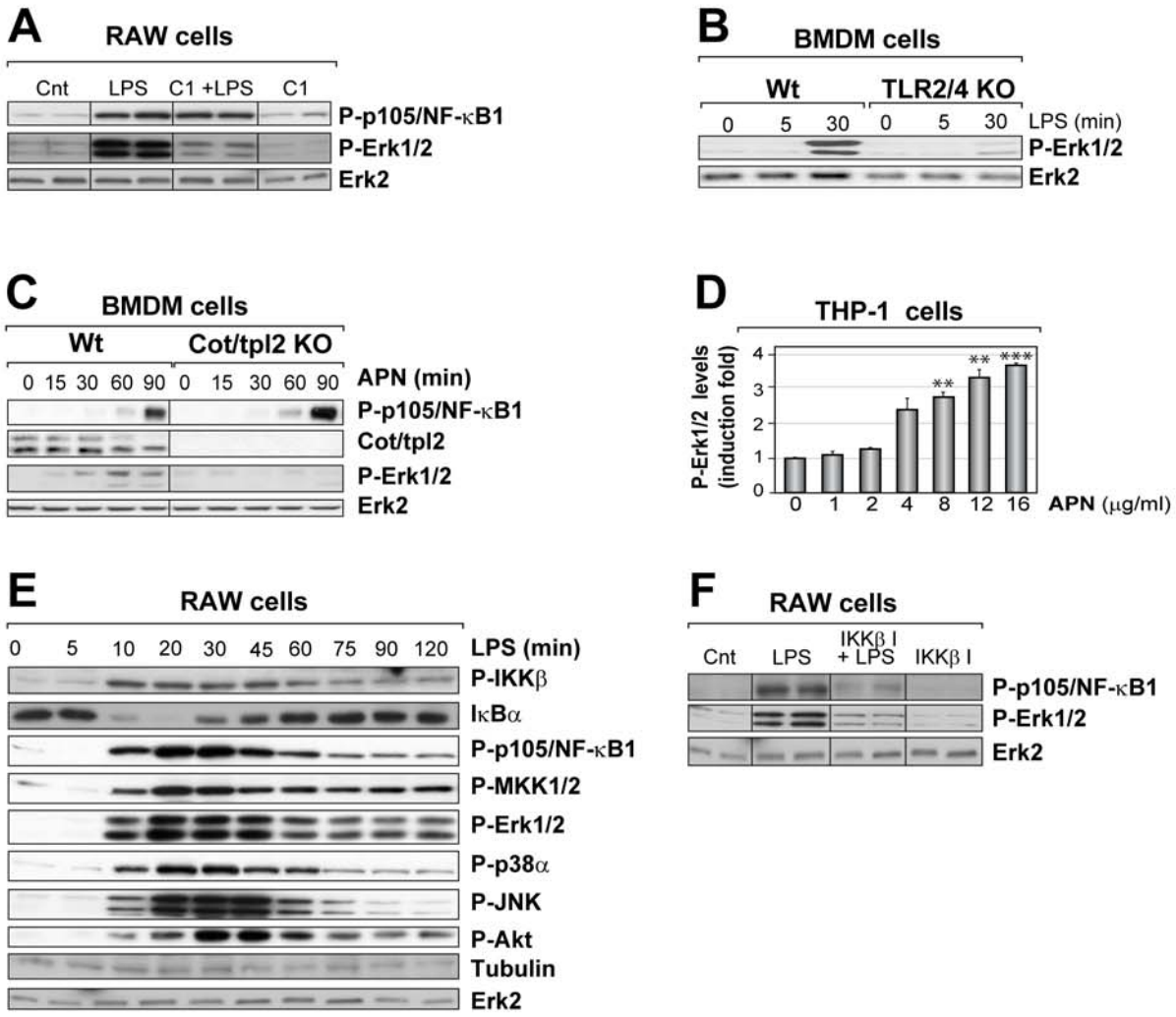


Figure S1. Erk1/2 activation in LPS and Adiponectin activated macrophages. A) Cot/tpl2 inhibitor blocks Erk1/2 activation by LPS: RAW cells were treated with LPS (300 ng/ml) for 30 min in the absence or presence of 5 μM Cot/tpl2 inhibitor (C1), and the levels P-p105/NF-κB1, P-Erk1/2 and Erk2 were measured in Western blots. **B)** TLR2/4 are required to Erk1/2 stimulation: Wt and TLR2/4 KO BMDM were incubated with LPS (300 ng/ml) and at the indicated times, the levels of P-Erk1/2 and Erk2 were measured in Western blots. **C)** Cot/tpl2 is degraded upon APN-stimulation: Wt and Cot/tpl2 KO BMDM were stimulated with APN (12 μg/ml) at the times indicated and the levels P-p105/NF-κB1, Cot/tpl2, P-Erk1/2 and Erk2 were measured in Western blots. **D)** Erk1/2 is phosphorylated after APN stimulation of THP-1 human macrophages: Dose response curve was performed incubating differentiated THP-1 macrophages with different amounts of APN for 1 h and the levels of P-Erk1/2 were measured by Western blots. The total Erk2 levels were used as loading control. Graph shows the mean of the induction fold of P-Erk1/2/Erk2 ± SD of 3 independent experiments performed in triplicate. **E)** MAPKs activation in LPS-stimulated RAW macrophages. RAW cells were incubated with LPS (300 ng/ml) and at the indicated times, the levels of P-IKKβ, IκBα, P-p105/NF-κB1, P-MKK1/2, P-Erk1/2, P-JNK, P-p38α and P-Akt, were measured in Western blots. The total Erk2 and tubulin levels were determined as protein loading controls. **F)** IKKβ inhibitor block LPS mediated Erk1/2 and p105/NF-κB1 phosphorylation: RAW cells were incubated for 1 h with 10 μM of the IKKβ inhibitor (IKKβ I) B1605906 and then stimulated for 30 min with LPS (300 ng/ml). At the indicated times, the levels P-p105/NF-κB1, P-Erk1/2 and Erk2 were measured in Western blots.(A, B, C, E and F): One representative experiments of the 3 performed is shown.

SUPPLEMENTARY TABLE 1

Real-time PCR primers

Genes	Forward Prmier	Reverse Primer
18 S	acggaagggcaccaccagga	caccaccacccacggaatcg
Adipo R1	acgttggagagtcatcccgtat	tcttgaagcaagcccgaaag
Adipo R2	agcctctatatcaccggagctg	gctgatgagagtgaaaccagatgt
Adora2b	gagctccatctttagcctcttg	tgtcccagtgaccaaacctt
CCL3	tgcccttgctgttcttctct	gtggaatcttccggctgtag
CCR2	acctgtaaatgccatgcaagt	tgtcttccatttcctttgatttg
CCR5	tgatgttagatttgtacagctctcct	tgacccttgaaaatccatcc
CCR7	atttctacagccccagagc	agcacactggaaaatgaca
CLEC10A	aaaacccaagagcctggtaaa	aggtgggtccaagagaggat
COX2	gatgctcttccgagctgtg	ggattggaacagcaaggattt
CXCL1	agactccagccacactccaa	tgacagcgcagctcattg
CXCL2	aaaatcatccaaaagatactgaacaa	ctttggttcttccgttgagg
CXCL3	ccccaggcttcagataatca	tctgatttagaatgcaggtcctt
CXCL9	cttttcctcttgggcatcat	gcatcgtgcattccttatca
DUSP2	gaagataaccagatggtggagataa	ccccactattcttcaccgagt
HSPA1A	ggccagggctggattact	gcaaccaccatgcaagatta
IFITM6	ccggatcacattacctggtc	catgtcgcccaccatctt
IL-10	tcctcctccagctcttacctc	tggctttccctaggactctct
IL-19	tggagaacctcaggagcatt	gaatgtcagcaggttgttgg
IL-1β	agctggatgctctcatcagg	agttgacggaccccaaaag
IL-27	catggcatcacctctctgac	aagggccgaagtgtggta
IL-6	gctaccaaactggatataatcagga	ccaggtagctatggtactccagaa
MRC1	ccacagcattgaggagtttg	acagctcatcatttggctca
PTPRO	ccagagctgagctgttgtga	cgtatttctggagcaacg
SAA2	ttcatttattggggaggcttt	gccagcttccttcatgtcag
SERPINF1	aggatcgaggtaaacgagagc	gcgggctgagatgacaaa
ST6GAL1	cttcccgattgagagagctg	ccggggcactgataacttc
TLR13	ctatgtgctaggagcttctgagag	aggaagcagagaaccaggaa
TNFSF9	cgccaagctactggctaaaa	cgtacctcagaccttgagataggt
TNFα	cgatcaccccgaagttcagta	ggtgcctatgtctcagcctctt

Mouse siRNA sequences

Genes	Sense Sequence	Antisense Sequence
Adipo R1	GGCUGAAAGACAACGACUAtt	UAGUCGUUGUCUUUCAGCCag
Adipo R2	GGCCCAUCAUGCUAUGGAAtt	UUCCAUAGCAUGAUGGGCCtg

SUPPLEMENTARY TABLE 2

TOP up-regulated genes in APN-stimulated macrophages

Syste. Name	Gene ID	Symbol	Genename	logFC	AveExpr	t	P.Value	adj.P.Val
NM_011198	19225	Ptgs2 (COX2)	prostaglandin-endoperoxide synthase 2	6,352043967	11,52953275	33,79129697	3,59E-15	2,53E-12
NM_031168	16193	Il6	interleukin 6	6,000878628	11,10628326	24,40672344	3,68E-13	4,36E-11
NM_203320	330122	Cxcl3	chemokine (C-X-C motif) ligand 3	5,966238356	8,89882688	31,8187462	8,48E-15	4,14E-12
NM_011314	20209	Saa2	serum amyloid A 2	5,961344489	6,868582898	18,37974003	1,97E-11	8,18E-10
NM_007413	11541	Adora2b	adenosine A2b receptor	5,401339105	10,48105957	36,55955175	1,16E-15	1,16E-12
NM_009404	21950	Tnfsf9	tumor necrosis factor (ligand) superfamily, member 9	5,30470983	13,09604753	32,34183867	6,72E-15	3,71E-12
NM_010479	193740	Hspa1a	heat shock protein 1A	5,297743688	13,0488706	18,28392634	2,12E-11	8,63E-10
NM_008361	16176	Il1b	interleukin 1 beta	5,203296802	12,47224137	29,8445052	2,11E-14	6,61E-12
NM_008176	14825	Cxcl1	chemokine (C-X-C motif) ligand 1	5,201398371	10,8447446	45,34629886	5,30E-17	2,41E-13
NM_010090	13537	Dusp2	dual specificity phosphatase 2	5,200674643	13,20067756	14,60276178	4,67E-10	9,83E-09
NM_008871	18787	Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	5,152405752	11,8436854	19,7220501	7,37E-12	3,82E-10
NM_007719	12775	Ccr7	chemokine (C-C motif) receptor 7	5,122528758	11,64102894	46,90530013	3,26E-17	2,02E-13

Data show the 12 most up-regulated genes in macrophages after 18 h of stimulation with adiponectin. Data are the mean from 3 different arrays performed with 3 different sets of RNA. **log FC** number of change in log 2; **AveEXpr**. mean of log2 of the probe in all the arrays and in all the channels; **t-**moderated t-statistic; **adj P value** express the false positive rate.

SUPPLEMENTARY TABLE 3

TOP down-regulated genes in APN-stimulated macrophages

Syste. Name	Gene ID	Symbol	Genename	logFC	AveExpr	t	P.Value	adj.P.Val
NM_009915	12772	Ccr2	chemokine (C-C motif) receptor 2	-4,398797786	9,494349205	-27,60705767	6,41E-14	1,36E-11
NM_001033632	213002	Ifitm6	interferon induced transmembrane protein 6	-4,120617805	12,53211445	-25,8639511	1,62E-13	2,46E-11
NM_205820	279572	Tlr13	toll-like receptor 13	-4,019050147	13,27571527	-44,92576503	6,06E-17	2,41E-13
NM_008625	17533	Mrc1	mannose receptor, C type 1	-3,934612391	11,29412112	-29,05066795	3,10E-14	8,27E-12
NM_001204252	17312	Clec10a	C-type lectin domain family 10, member A	-3,857729685	9,580982254	-18,32989597	2,04E-11	8,42E-10
NM_009917	12774	Ccr5	chemokine (C-C motif) receptor 5	-3,714317504	11,63332335	-13,94747493	8,70E-12	1,64E-10
NM_011216	19277	Ptpro	protein tyrosine phosphatase, receptor type, O	-3,477394243	11,54772294	-27,63452455	6,32E-14	1,34E-11
NM_145933	20440	St6gal1	beta galactoside alpha 2,6 sialyltransferase 1	-3,424432413	8,121639616	-16,80630914	6,80E-11	2,12E-09

Data show the 8 most down-regulated genes in macrophages after 18 h of stimulation with adiponectin. Data are the mean from 3 different arrays performed with 3 different sets of RNA. **log FC** number of change in log 2; **AveEXpr**. mean of log2 of the probe in all the arrays and in all the channels; **t-**moderated t-statistic; **adj P value** express the false positive rate.

Discusión

Cot/tpl-2 es una quinasa incluida en el grupo de las MAP3K. Es la única que activa Erk1/2, vía MKK1/2, en respuesta a la activación de la superfamilia de receptores TLR/IL-1R, los receptores de la familia del TNFα (CD40, TNFR1 y TNFR2) [11, 36, 39, 42, 75, 83, 84, 159], así como en la activación del FcγR y del receptor 1 activado por proteasas [65, 99]. Durante el desarrollo de esta Tesis Doctoral hemos establecido el papel de Cot/tpl-2 (MAP3K8) en la inflamación estéril inducida por una sobredosis de APAP. Asimismo se ha estudiado la señalización intracelular inducida tras la estimulación de macrófagos por DAMPs, LPS y APN y la implicación de Cot/tpl-2 en la expresión de genes relacionados con el proceso de activación de los macrófagos.

1. Activación de Cot/tpl-2--MKK1/2--Erk1/2 por señales extracelulares: implicación en el estado de fosforilación de otras proteínas.

La familia de receptores TLRs actúan como sensores de las infecciones de patógenos por su capacidad de reconocer y activarse en respuesta a PAMPs. Por ejemplo, el LPS activa el TLR4 [130, 148, 179]. En los últimos años se ha demostrado que los TLRs y otros receptores incluidos en la superfamilia de los PRRs, también reconocen moléculas propias endógenas del organismo que en condiciones fisiológicas no están en el mismo microambiente que los TLRs. Así, los TLRs son también sensores del daño celular ya que responden a DAMPs [153].

La activación del eje MKK1/2-Erk1/2 vía Cot/tpl-2 por la estimulación de los TLRs es independiente del TLR que se active y del adaptador intracelular (MYD88 o TRIF) responsable de transducir la señal [85]. La activación fisiológica de Cot/tpl-2 ha sido ampliamente estudiada en macrófagos derivados de medula ósea, peritoneales o líneas celulares establecidas. La estimulación con Zymosan (TLR2/6), Poly I:C (TLR3), LPS (TLR4), Loxoribine (TLR7) o dsRNA (TLR8) de macrófagos Cot/tpl-2 KO muestran una falta de activación de Erk1/2 en contraposición a lo observado en macrófagos que si expresan Cot/tpl-2 [11, 23, 108, 109]. La estimulación de células dendríticas y linfocitos B con CpG-DNA implica la estimulación de Cot/tpl-2 tras la activación del TRL9 [11, 108, 177], aunque no se puede descartar que otras MAP3K además de Cot/tpl-2 regulan la vía Erk1/2 en respuesta a CpG-DNA [83]. Por otro lado también se ha demostrado que citoquinas proinflamatorias como el TNF o IL-1 activan Erk1/2 vía Cot/tpl-2.

Nosotros, en esta Tesis Doctoral hemos demostrado que la APN, hormona implicada en procesos biológicos en principio no encuadrados dentro de la respuesta inmune también regula la actividad de Cot/tpl-2 en macrófagos. La APN tiene como función principal

sensibilizar los efectos de la insulina a través de los receptores AdipoR1 y AdipoR2 [29, 81, 214]. Estos receptores constituyen una familia propia de 7 dominios trans-membrana, donde el dominio N-terminal citoplasmático interacciona con la proteína adaptadora APPL1 [118, 185], mediando el incremento en la captación de glucosa y la oxidación de ácidos grasos por la activación de PPARα, p38 MAPK y AMPK [211, 213].

Los receptores AdipoR1 y AdipoR2 se expresan principalmente en músculo esquelético e hígado, tejidos dianas en los que actúa la APN [185]. Tras la estimulación con APN, hepatocitos Cot/tpl-2 KO y Wt mostraban niveles de P-Erk1/2 muy parecidos y sin variaciones significativas, lo que parece indicar que APN no regula la activación de Cot/tpl-2, ni incrementa la fosforilación de Erk1/2, en este tipo celular. La transfección de células RAW 264.7 con diferentes siRNAs contra los receptores AdipoR1 y AdipoR2, por separado o juntos, conduce a la disminución en la expresión de sus mRNAs; pero su estimulación con APN no implica un cambio en los niveles de P-Erk1/2, indicando que la activación de los macrófagos por APN es AdipoR1 y AdipoR2 independiente. Estos resultados concuerdan con los obtenidos recientemente en células dendríticas estimuladas con APN, donde la expresión de IL-12 es independiente de la expresión de estos receptores [80].

Todos los receptores de la superfamilia TLR/IL-1 que activan Cot/tpl-2, contienen un dominio TIR que transducen la señal y todos a excepción de TLR3, que señaliza a través del adaptador TRIF, activan a IKKβ a través del eje MYD-88-TRAF-TAK1 [23, 101]. Sin embargo se sabe que en otras condiciones de activación celular, estimulación con TNFα o con CD40L, otras proteínas están implicadas en la activación de Cot/tpl-2. Tras su activación, TNFR1 recluta el complejo RIP-TRAF2-TAK1 que subsecuentemente activa a IKKβ [201]. Mientras que otros miembros de esta familia de receptores como TNFR2 y CD40 reclutan directamente diferentes proteínas TRAF y subsecuentemente a TAK1 [63]. Además, TAK1 puede ser activado por otras proteínas adaptadoras [168, 202]. Por otro lado, la posibilidad de que otra MAP3K además de TAk1 pueda activar Cot/tpl-2 no puede ser descartada [1]. Asimismo, Cot/tpl-2 también se puede activar por la estimulación del receptor 1 activado por proteasas, receptor acoplado a proteina G y de FcγR, pero el mecanismo aún se desconoce [65, 99].

Estudios estructurales realizados en el dominio globular del extremo C-terminal de la APN, muestran una secuencia de aminoácidos muy parecida a la proteína C1q, que participa en el reconocimiento de superficies microbianas y en los complejos anticuerpo-antígeno en la vía clásica del complemento. La conformación tridimensional de APN es similar a la del colágeno VIII, la proteína de unión a Manosa y el TNFα [72, 172, 212, 213]. Estructuras cristalográficas a una resolución de 2.1 Å muestran el gran parecido entre la parte globular de la APN y el TNFα

[174]. Los experimentos aquí mostrados indican que APN no activa Cot/tpl-2 en macrófagos vía TLR2 o TLR4, los dos receptores mayoritarios de los TLRs que se expresan en la superficie de la membrana plasmática. Sin embargo, la posibilidad de que APN se una a un receptor de la familia de TNFα además de a otro tipo de receptor en macrófagos queda por dilucidar.

En los últimos años ha crecido la evidencia de que algunas moléculas endógenas, al encontrarse en un medio no fisiológico, tienen la capacidad de alertar de dicha disfunción tisular gracias a su capacidad de activar a los receptores que reconocen PAMPs, entre los que se encuentran los TLRs. Las moléculas endógenas con dicha capacidad han sido denominadas DAMPs. Como consecuencia de la necrosis hepática producida por una sobredosis de APAP se liberan al medio extracelular dichas moléculas, que sirven como mensajeros del daño celular para las células vecinas [153]. La unión de los DAMPs a los diferentes TLRs, como el DNA mitocondrial al TLR9 en neutrófilos humanos y células endoteliales o las HMGB1 en macrófagos, promueve la activación de las MAPKs p38, Erk1/2 y JNK [28, 178, 217]. Los experimentos mostrados en esta Tesis, realizados tanto en macrófagos peritoneales como en macrófagos derivados de médula ósea deficientes en Cot/tpl-2 demuestran que esta MAP3K participa en la activación de Erk1/2 y de JNK, mediada por DAMPs. Nuestros datos muestran como en macrófagos estimulados con DAMPs se producen dos picos de activación de Erk1/2 diferentes a 5 min y a 30 min. Mientras que la activación de Erk1/2 a 5 min se mantiene constante en ambos tipos celulares, la deficiencia de Cot/tpl-2 en macrófagos reduce significativamente la fosforilación de Erk1/2 que se observa a los 30 min. Además, la inhibición de la actividad IKKβ, necesaria para la activación del eje Cot/tpl-2- Erk1/2, bloquea el segundo pico de fosforilación de Erk1/2. Estos datos indican que el segundo pico de activación de P-Erk1/2 está mediado por Cot/tpl-2.

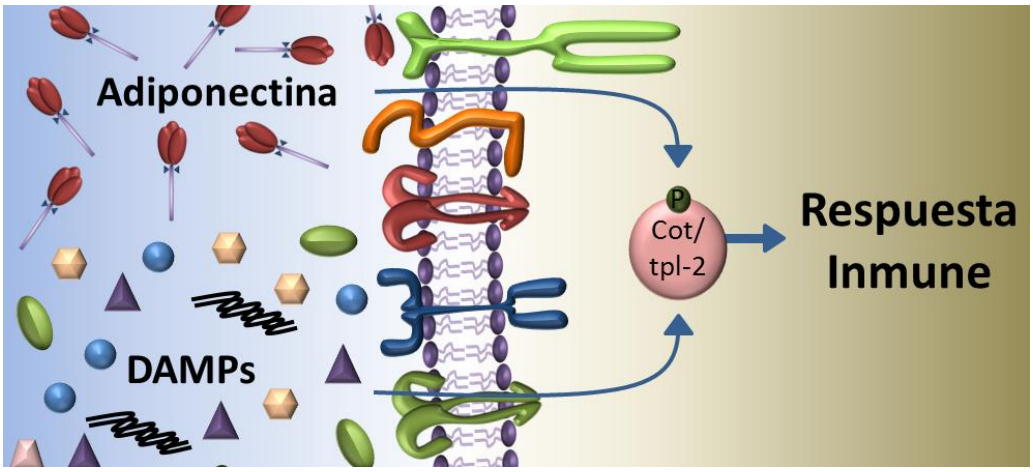


Imagen 9: Esquema representativo que muestra que DAMPs y APN activan la respuesta inmune en macrófagos a través de Cot/tpl-2.

Tras la estimulación con DAMPs, se observa una reducción en la activación de JNK en macrófagos Cot/tpl-2 KO, que podría estar mediada por la falta de activación de su MAP2K, MKK4 [35], sin embargo la deficiente expresión de Cot/tpl-2 en macrófagos no afecta a la fosforilación de p38 ni de p105-NFκB. Se ha descrito que Cot/tpl-2 puede activar también otras MAPKs dependiendo del tipo celular y del estímulo. Así, Cot/tpl-2 media la activación de Akt y de p70 S6K en macrófagos estimulados con LPS y la activación de p38α y JNK en MEFs, células B, células dendríticas y macrófagos estimulados con TNFα, CD40L, CpG o IL-1β [36, 54, 83, 109].

En esta Tesis Doctoral demostramos que Cot/tpl-2 también regula la traducción de mensajeros debido a su capacidad de activar Erk1/2. Así, en respuesta a la activación de los TLRs, Cot/tpl-2--MKK1/2--Erk1/2 regula la traducción de mensajeros modificando el estado de activación de proteínas implicadas en la vía de PI3K-Akt-mTORC1.

La deficiencia de Cot/tpl-2 en macrófagos no afecta a la fosforilación de Akt en T308, indicando que Cot/tpl-2 no regula la activación de PI3K en macrófagos estimulados con LPS, pero sí presentan una fosforilación de Akt en S473 deficiente en comparación con los macrófagos Wt. Además, Akt requiere estar fosforilada en ambos residuos T308 y S473 para fosforilar a FOXO1 en T24 y efectivamente dicha fosforilación se ve comprometida en macrófagos Cot/tpl-2 KO con respecto a los macrófagos Wt. La fosforilación de Akt en S473 está catalizada por mTORC2 y nuestros datos demuestran que otra diana de mTORC2 como es la S422 de SGK1 [56], también está reducida en los macrófagos Cot/tpl-2 KO en respuesta a LPS. Por el momento se desconoce el mecanismo por el cual mTORC2 se activa en respuesta a distintas señales extracelulares. Nuestros datos sugieren que Cot/tpl-2 puede mediar la activación de este complejo en respuesta a la estimulación de TLRs en macrófagos. Serán necesarios futuros experimentos para determinar el mecanismo por el cual Cot/tpl-2 podría regular la activación de mTORC2.

Mientras que mTORC2 es un efector importante de la regulación de las vías de crecimiento celular y supervivencia, mTORC1 es el encargado de la integración de las señales proliferativas de la vía de PI3K con la vía sensora energética. Dichas señales van a determinar si se comienza o no la traducción de mensajeros [197].

Para la activación del complejo mTORC1, vía PI3K, es suficiente la fosforilación de Akt en T308. Akt activa, fosforila e inhibe la función de TSC1-TSC2, permitiendo así la activación del complejo mTORC1 [162]. Está descrito que señales proliferativas que activan Erk1/2 vía RAF también tienen la capacidad de inhibir la actividad de TSC1-TSC2. Erk1/2 activa por

fosforilación en T573 a RSK y esta a su vez es capaz de inhibir a TSC1-TSC2 por fosforilación en S1798. Así, Erk1/2 también tiene la capacidad de activar al complejo mTORC1 [3, 114]. Nosotros aquí demostramos que Cot/tpl-2 también tiene la capacidad de regular la fosforilación de RSK en T573 y de TSC1-TSC2 en S1798.

El paso limitante en la traducción de mRNAs es la iniciación, donde el ribosoma se une al mRNA y busca el codón de inicio. La mayoría de los mRNAs eucariotas poseen una estructura Cap en el extremo 5´del mRNA. La traducción de estos mensajeros está mediada por la unión de eIF4E a la estructura Cap, seguido por la unión de otra serie de proteínas que constituyen el complejo de iniciación de la traducción. eIF4E está asociado a la proteína inhibidora 4E-BP1, interacción que impide la unión de proteínas requeridas para el inicio de la traducción. El control regulador de este proceso se ejerce facilitando la disociación de 4E-BP1 de eIF4E, tras la fosforilación de 4E-BP1 en S65 por mTORC1 [16], siendo el paso limitante en la traducción de mRNAs Cap-dependientes [155]. Los datos aquí mostrados indican que la deficiencia de Cot/tpl-2 en macrófagos da lugar a una fosforilación ineficiente de 4E-BP1 en respuesta a LPS. Estos datos sugerirían una deficiente traducción de los mRNA Cap-dependientes en macrófagos Cot/tpl-2 KO tras la estimulación con LPS, hecho que se discutirá posteriormente.

Nuestro grupo ya había demostrado anteriormente que en macrófagos estimulados con distintos PAMPs, como LPS (TLR4); Zymosan o Poly I:C, que activan respectivamente TLR2 y TLR3 [109], la fosforilación de otro sustrato directo de mTORC1, p70-S6K1, también presentaba una fosforilación reducida en los macrófagos Cot/tpl-2 KO con respecto a los Wt. Además, mTORC1 es capaz de activar la proteína de la subunidad pequeña del ribosoma S6 en los residuos S235/236 y de la quinasa del factor de elongación eEF2, eEF2K, en S366 [114, 155]. Estas dianas son también sustratos de RSK [3], por lo que la deficiencia en la fosforilación de RSK y p70-S6K1 en los macrófagos Cot/tpl-2 KO correlaciona con una menor fosforilación de eEF2K y S6.

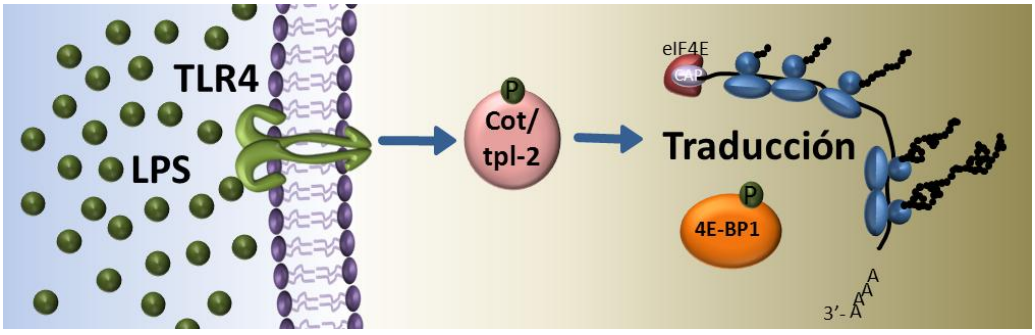


Imagen 10: Cot/tpl-2 media el inicio de la traducción en macrófagos estimulados con LPS.

2. Influencia de Cot/tpl-2 en la producción de mediadores inflamatorios en macrófagos. Regulación de la expresión génica, de sus niveles de traducción y de secreción.

La producción de mediadores inflamatorios y su liberación al medio contribuyen al reclutamiento y activación de células inflamatorias, que a su vez producen la liberación de nuevos mediadores, aumentando las concentraciones locales en respuesta a un estímulo inductor apropiado y modulando así el evento inflamatorio [121]. Los macrófagos son células plásticas que se adaptan a diferentes microambientes tisulares asumiendo una gama de diferentes fenotipos. En consecuencia, pueden presentar ya sea un fenotipo pro- (M1) o anti-inflamatorio (M2) [45]. Por otra parte, la APN se ha descrito como capaz de modular a células del sistema inmune a un fenotipo determinado según las condiciones de estimulación, de hecho APN ha sido propuesta como capaz provocar una respuesta tanto pro-inflamatoria como anti-inflamatoria en macrófagos [34, 47, 80, 92, 94, 141]. Recientemente se ha descrito que en linfocitos CD4, la APN promueve un programa pro-inflamatorio resultando en una polarización Th1 y Th17; además de conducir a un fenotipo M1 pro-inflamatorio en macrófagos [80]. Datos presentados en esta Tesis Doctoral apoyan el hecho de que la estimulación de macrófagos con APN promueve un fenotipo pro-inflamatorio M1. Otros autores han descrito que APN tiene una acción anti-inflamatoria con la subsiguiente desensibilización frente a estímulos pro-inflamatorios como el LPS [34, 94, 141].

Tabla 2: Mediadores inflamatorios regulados *in vitro* por Cot/tpl-2 según estímulo y tipo celular. BMDM: macrófagos derivados de médula ósea; BMDC: células dendríticas derivadas de médula ósea; KC: células de Kupffer; HSC: células estrelladas hepáticas; PM: macrófagos peritoneales. *: niveles de mensajero; ˜: niveles de proteína.

Señal Inflamatoria	Tipo Celular	Citoquinas	Quimioquinas	Refs
LPS	BMDM, BMDC, KC,HSC	↑IFNβ* ˜,↑IFNγ*,↓TNFα*˜ ↑IL-12* ˜,↑IL-12β* ˜ ↓IL-6*,↓IL-10 * ˜ ↓IL-1β* ˜, ↓IL-23*	↓CCL2*, ↑CCL5*, ↓CCL7* ↑CCL8*, ↓CXCL1*, ↓CXCL2* ↓CXCL3*, ↑CXCL9* ↑CXCL10*, ↑CXCL13*	[10, 83, 109, 125, 152]
Zymosan	BMDM, BMDC	↔TNFα ˜		[125]
Poli I:C	BMDM, BMDC	↓TNFα ˜		[125]
IL-1	Hela	↓IL-8*	↓CCL4*	[159]
CpG	BMDM, BMDC	↑IFNβ˜, ↑IL-12 ˜, ↓TNFα˜ ↑IL-12β ˜, ↓IL-10 ˜		[83, 125]
APN	BMDM	↓IL-1α* ˜, ↓IL-1β* ˜, ↑IL-6* ˜ ↑IL-12* ˜, ↓TNFα* ˜ ↓IL-10* ˜, ↓IL-19*, ↑IL-27*	↑CCL2˜, ↓CCL3* ˜ ↑CXCL1* ˜, ↓CXCL2* ↓CXCL3*, ↑CXCL9*	
DAMPs	PM	↓IL-1α ˜, ↓IL-1β ˜, ↓IL-6 ˜ ↓TNFα ˜, ↓IL-10 ˜		

El estudio global de la expresión génica nos permite averiguar los cambios existentes en las diferentes vías en unas condiciones determinadas. Nuestros datos demuestran que la estimulación de macrófagos con 12 µg/ml de APN aumenta la respuesta inflamatoria. El análisis de la modulación de la expresión de genes tras la estimulación con APN revela que las dos funciones que más se modulan son la respuesta inflamatoria y la respuesta a agentes infecciosos siendo el grupo interacción citoquina–receptor el más afectado según el programa "Kyoto Encyclopedia of Genes and Genomes".

Ya se había descrito anteriormente un papel de Cot/tpl-2 en la producción de mediadores inflamatorios inducidos por otras señales extracelulares tanto en macrófagos como en otros tipos celulares [10, 83, 109, 159, 161, 175]. El análisis de los datos del array llevado a cabo tras la estimulación de macrófagos Wt y Cot/tpl-2 KO con APN indica que muchos de los mediadores inflamatorios descritos en la Tabla 3 regulados por Cot/tpl-2 en diferentes tipos celulares y con distintos estímulos, también se modulan tras la estimulación de macrófagos con APN. Vale la pena resaltar que Cot/tpl-2 reprime la expresión de los mensajeros de IL-27, IL-6, e IL-12, al igual que ocurre con esta última en macrófagos estimulados con LPS [109]; además también de reprimir la expresión de CCL3 y CXCL9 quimioquinas pertenecientes a la familia de CCL5 y CXCL13, respectivamente, que también son reprimidas tras la estimulación de macrófagos por LPS. [10].

Seis de los 12 genes cuya expresión está más aumentada tras la estimulación de macrófagos con APN están englobados en el grupo de interacción citoquina–receptor. Cot/tpl-2 también controla la expresión de genes regulados por APN con funciones muy variadas y así, Cot/tpl-2 es esencial para la expresión de Adora2b, HSPA1a, TNFSF9 y SAA2. Ésta última es una proteína implicada en la formación de células espumosas [86, 123], que también se encuentra altamente expresada en condiciones de inflamación aguda tras la exposición a LPS. Por otra parte, los 8 genes cuya expresión está más disminuida tras la estimulación de macrófagos con APN son totalmente independientes de Cot/tpl-2.

Recientemente se ha descrito la inhibición de la expresión de algunos genes como COX2, IL-6 e IL-12β por un sistema de retroalimentación negativo mediado por la vía de la IL-10 [115, 134, 138]. El uso de un anticuerpo específico para IL-10 bloquea la fosforilación de STAT3 en macrófagos Wt tras la estimulación con APN. Por el contrario, macrófagos Cot/tpl-2 KO, que no secretan altos niveles de IL-10, no muestran niveles significativos de P-STAT3. El análisis de expresión de COX2, IL-6 e IL-12β en macrófagos Wt estimulados con APN en presencia de anti-IL-10, muestra un pequeño aumento significativo de la expresión de los genes con respecto a las muestras no tratadas con anti-IL-10.

La modulación de la expresión de los mensajeros de los mediadores inflamatorios por Cot/tpl-2 se correlaciona con los cambios en los niveles de producción de estas proteínas. Así la medida de los niveles de estos mediadores en el medio de macrófagos estimulados con APN demuestra que Cot/tpl-2 es esencial para la producción de TNFα, IL-1α, IL-1β, IL-10 y CXCL1; mientras que no lo es para la producción de IL-12 y CCL2. Estos datos confirman resultados descritos anteriormente como que Cot/tpl-2 es necesaria para la producción de TNFα e IL-1β, y por el contrario, no lo es para la producción de IL-12 y IL-27 en macrófagos y células dendríticas estimuladas con LPS [125] o en el modelo inducido por Zymosan [175].

Mientras que la deficiencia de Cot/tpl-2 no afecta a los niveles de expresión de CCL3, la producción de este mediador se ve aumentada en el medio de macrófagos Wt estimulados con APN con respecto a los macrófagos Cot/tpl-2 KO. De igual forma, aunque los niveles de expresión del mRNA de IL-6 se encuentran aumentados en macrófagos deficientes en Cot/tpl-2, la producción de esta citoquina es equiparable entre macrófagos Wt y Cot/tpl-2 KO tras la estimulación con APN. Datos de esta Tesis Doctoral que discutiremos posteriormente sostienen que Cot/tpl-2 es capaz de actuar a diferentes niveles post-transcripcionales, aumentando la tasa de traducción de diferentes mediadores inflamatorios en macrófagos estimulados con LPS, por lo que no se puede excluir que Cot/tpl-2 actúe de la misma forma en la traducción de distintos mensajeros en macrófagos estimulados con APN.

Muchas de las funciones de la respuesta inflamatoria, como la fagocitosis, se favorecen tras la activación de los macrófagos por las citoquinas IL-1 y TNFα [119, 198].Los datos existentes en la literatura respecto a la modulación de la fagocitosis por APN indican que esta hormona afecta negativamente al proceso y así se ha publicado que APN inhibe la fagocitosis de *E. Coli* en neutrófilos mediante la inhibición de PKB y la MAPK Erk1/2 [160], así como la fagocitosis de células apoptóticas por macrófagos humanos, que por el contrario es modulada positivamente en macrófagos estimulados con LPS [163]. Nosotros hemos observado que mientras que los niveles basales de captación de bolas recubiertas de Zymosan en macrófagos Wt y deficientes en Cot/tpl-2 son muy parecidos, Cot/tpl-2 es necesaria para la fagocitosis en macrófagos estimulados con APN, quedando reducida casi a la mitad esta función en macrófagos Cot/tpl-2 KO. Dado que el LPS media la polarización a un fenotipo pro-inflamatorio M1 en macrófagos, y está bien documentado que lo hace utilizando el eje IKKβ--p105-NFκB1--Cot/tpl-2--MKK1/2--Erk1/2 que es el mismo que utiliza APN, cabe la posibilidad de que APN favorezca la fagocitosis mediante el mismo mecanismo, aunque este extremo ha de ser estudiado en más detalle.

3. Regulación de la biosíntesis de proteínas y del control de mensajeros por Cot/tpl-2 en respuesta a LPS

Macrófagos Cot/tpl-2 KO presentan una fosforilación deficiente de 4E-BP1 con respecto a los Wt tras la activación de distintos TLRs, siendo dicha fosforilación clave para la disociación del complejo 4E-BP1--eIF4E. Una vez que eIF4E se ha disociado del complejo y para que se inicie la traducción de los mRNAS Cap-dependientes es necesario que eIF4E se una a la estructura Cap, formándose el complejo de iniciación de la traducción activo que regula la unión del ribosoma al mRNA [114, 155]. Tras el análisis de la eficiencia de la traducción Cap-dependiente de la luciferasa de Renilla y de la traducción IRES-dependiente de la luciferasa de luciérnaga, hemos comprobado que la falta de disociación de eIF4E y 4E-BP1 en los macrófagos Cot/tpl-2 KO correlaciona con una disminución en la traducción Cap-dependiente, hecho que se hace especialmente evidente tras la estimulación con LPS.

La separación de complejos de traducción en gradientes lineales de sacarosa permite el análisis de la condición física general de la síntesis proteica, así como la investigación detallada de las funciones fisiológicas desempeñadas por factores individuales de la maquinaria de traducción [120]. Nuestros resultados demuestran que Cot/tpl-2 no modifica el perfil general de polisomas, pero si el reclutamiento de mRNAs específicos a ribosomas en respuesta a LPS. Estudios previos han determinado que pese a que la unión de eIF4E a la estructura Cap es un factor necesario para la traducción eficiente de la mayoría de mRNAs, la disociación del complejo 4E-BP1-- eIF4E sólo afecta levemente a la tasa de traducción de proteínas general de las células [16]. Sin embargo, para la traducción de mRNAs con estructuras secundarias complicadas en la región 5´ es necesaria la formación del complejo de iniciación activo eIF4F con actividad helicasa que desenrolla las estructuras secundarias del mRNA, permitiendo que el ribosoma se una a la estructura Cap y encuentre el codón de inicio [155]. En este contexto, el análisis de mRNAs específicos en respuesta a LPS, demuestran que la deficiencia de Cot/tpl-2 afecta severamente a la traducción de los mensajeros de IL-6, TNFα y KC, que tienen estructuras secundarias complejas [67, 186].

Muchas de las proteínas ribosomales y proteínas que regulan la maquinaria de traducción, como los factores de elongación, vienen codificados por los denominados 5´ TOP mRNAs, muy sensibles a la activación de mTORC1 [154]. Los factores de elongación eEF1α y eEF2 estudiados, transcritos 5´ TOP, muestran una traducción inhibida en macrófagos Cot/tpl-2 KO en respuesta a LPS, y en macrófagos tratados con UO 126, un inhibidor de la actividad Erk1/2. Destacar que los niveles de mRNA totales de eEF1α y eEF2 no están modulados ni por

LPS ni por Cot/tpl-2. Esto sugiere que Cot/tpl-2 modula tanto la iniciación como la elongación de la traducción gracias a su capacidad de activar Erk1/2.

La estabilidad de los mRNAs, junto a las tasas de transcripción dan como resultado los niveles totales de mRNAs. Dicha estabilidad se controla por interacciones coordinadas entre componentes estructurales del mRNA (elementos cis) y factores trans específicos. Los determinantes de estabilidad de los mRNAs más conocidos y eficientes son los elementos ricos en adenina y uridina (ARE) que modulan la estabilidad de los transcritos y/o su traducción [145]. Se ha demostrado que Cot/tpl-2 modula la expresión del mRNA de TNFα mediante un mecanismo en el que está implicado el elemento rico en AU presente en la región 3´ de este transcrito tras la estimulación con LPS [39]. Nuestros datos demuestran que Cot/tpl-2 induce tanto el aumento en la tasa de transcripción, de la traducción, así como de la estabilidad de los transcritos de IL-6, TNFα y KC en respuesta a LPS. Todos estos datos correlacionan con la mayor secreción de estos mediadores inflamatorios al medio extracelular tras la estimulación con LPS en los macrófagos Wt en comparación con los macrófagos Cot/tpl-2 KO.

4. Implicación de Cot/tpl-2 en la inflamación estéril causada por una sobredosis de Acetaminofén (APAP)

El hígado, uno de los órganos más importantes por su actividad metabólica, es el responsable de eliminar de la sangre las sustancias que puedan resultar nocivas para el organismo. Así, la sobredosis de APAP (paracetamol), uno de los analgésicos y antipiréticos más vendidos en Norte-América y Europa, es la primera causa de insuficiencia hepática aguda, causando aproximadamente el 50% de las lesiones hepáticas totales [18]. El APAP es metabolizado en el hígado a N-acetyl-p-benzoquinona imina (NAPQI) capaz de unirse al glutatión y eliminarse por la orina [126]. La sobredosis agota las reservas de glutatión por lo que se inicia una unión covalente de APAP a proteínas celulares. Estos eventos conducen a la interrupción de la homeostásis del calcio, la disfunción mitocondrial, y estrés oxidativo [124, 126] que finalmente, puede culminar en daño y muerte celular. La lesión hepática inducida por APAP está asociada con la inflamación estéril por su capacidad de generar DAMPs [5, 69]. Nuestros resultados demuestran la implicación de Cot/tpl-2 en la inflamación estéril, así como en el reclutamiento de células al foco inflamatorio y la producción de mediadores pro-inflamatorios *in vivo*.

En este contexto, ratones TLR9 KO, con menor producción de citoquinas inflamatorias, muestran mayor resistencia a la toxicidad inducida por APAP que los ratones que expresan

TLR9 [74]. Por otra parte, modelos *in vivo* con ratones Cot/tpl-2 KO muestran una reducción significativa de la inflamación, como en el modelo de pancreatitis aguda inducida por caerulina [196] o el modelo de inflamación periférica inducida por Zymosan [175]. Curiosamente, dos grupos distintos han propuesto un papel de Cot/tpl-2 distinto para el modelo experimental de colitis inducida por dextrano sulfato de sodio. Mientras que Cot/tpl-2 en un modelo más leve atenúa la respuesta inflamatoria [101], en un modelo mucho más agudo, Cot/tpl-2 promueve la tumorogénesis aumentando los niveles de mediadores inflamatorios [87]. Así se requiere un estudio más detallado para dilucidar el papel propuesto para Cot/tpl-2 en esta patología.

La sobredosis de APAP puede producir una necrosis hepática centrilobular, lo que desemboca en un fallo hepático [68] conduciendo a la liberación al torrente sanguíneo de marcadores de daño hepático como las transaminasas ALT y AST o de LDH [19]. Así, tras la administración i.p. de APAP a ratones Cot/tpl-2 KO se observa una tasa de supervivencia del 100% frente a una tasa de supervivencia del 12% al suministrar la misma dosis a ratones control. Además, cortes histológicos del hígado de ratones Cot/tpl-2 KO muestran una atenuación de la lesión hepática inducida por APAP cuando los comparamos con ratones Wt, que presentan altos niveles de necrosis y hemorragia. La congestión hepática es 3 veces superior en ratones Wt. Estos datos se correlacionan con los menores niveles de ALT y AST o de LDH en sangre de ratones deficientes en Cot/tpl-2 con respecto a los ratones Wt. La activación de JNK es también un marcador de lesión hepática [62], por ser un indicador del estrés celular y su activación se encuentra reducida en los hígados de ratones Cot/tpl-2 KO en comparación con los niveles observados en los ratones WT tras la administración de APAP.

La extravasación de células inflamatorias al área lesionada constituye uno de los primeros eventos en la defensa frente a agentes patógenos [32]. Para hacer frente al proceso patológico intervienen, en primer lugar, los neutrófilos y posteriormente, los macrófagos. Como está descrito, la lesión hepática inducida por APAP tiene como consecuencia el reclutamiento de leucocitos al hígado por los mediadores inflamatorios que son liberados por células que se encuentran en el hígado, entre las que caben destacar la células de Kupffer (KC) y las células estelares hepáticas (HSC) [5, 68, 69, 152]. El subsiguiente reclutamiento de leucocitos al hígado, a su vez da lugar a la generación de más mediadores inflamatorios, llegando en muchos casos a una respuesta inflamatoria exagerada y dañina para el propio organismo, debido a la sobredosis de APAP. En este contexto, el estudio de las diferentes poblaciones tras 18 h de tratamiento con APAP refleja una reducción del número total de neutrófilos, macrófagos y NKT en los hígados de ratones deficientes en Cot/tpl-2 respecto a los Wt tratados con APAP. Es de destacar, que la MFI de los marcadores de activación CD11a y

CD11b no varía en neutrófilos, observándose en macrófagos, una reducción en el marcador de activación CD11b [69] debido a la deficiencia de Cot/tpl-2, lo cual indica que hay un diferente grado de activación entre ambos.

Los DAMPs son factores endógenos, que en condiciones fisiológicas normalmente se encuentran en el interior de la célula y por lo tanto se encuentran ocultos al reconocimiento por receptores de la familia de los PRRs. Sin embargo, en condiciones de estrés o lesión, estas moléculas pueden ser liberadas al exterior del medio por muerte celular, provocando inflamación bajo condiciones estériles [70]. La sobredosis de APAP en el hígado provoca una alteración en los niveles de glutatión que conllevará a una alteración de procesos vitales para la célula. Finalmente aparece una disfunción mitocondrial y pérdida del balance energético, resultando en una necrosis celular y posterior inflamación [68]. Como está descrito, Cot/tpl-2 media la producción de la citoquina pro-inflamatoria TNFα y también de la citoquina anti-inflamatoria IL-10 en macrófagos estimulados con LPS [39, 83]. Tras el estudio de los niveles de mediadores inflamatorios en el medio de macrófagos peritoneales Cot/tpl-2 KO estimulados con DAMPs se observa una reducción en la producción de las citoquinas IL-1α, IL-1β, IL-6, y TNFα; incluso de la IL-10, citoquina que estaba aumentada en el suero de ratones deficientes en Cot/tpl-2 tras el tratamiento con APAP. El uso de los inhibidores PD0325901, específico para MKK1/2; y SP600125, específico para JNK, demuestran que la producción de estas citoquinas viene mediada por Cot/tpl-2 a través de la activación de Erk1/2.

Tabla 3: Efectos *in vivo* de la deficiencia de Cot/tpl-2 en inmunidad y respuesta inflamatoria (adaptado de [54])

Señal Inflamatoria	Fenotipo	Mediadores	Refs
LPS/D-Gal	Resistencia	Suero:↓ TNFα ,↓ IL-1β	[39, 125]
Zymosan	↓Inflamación *Intraperitoneal* ↓Reclut. Neutrófilos ↓Reclut. Macrófagos *Intraplantar* ↓Reclut. Neutrófilos ↓Hipernocicepción	*Intraperitoneal* Suero:↓TNFα *Intraplantar* ↓TNFα, ↓IL-1β, ↓ IL-6, ↓ G-CSF, ↓GM-CSF, ↓NGF, ↓ MCP-1,↔ MIP-α, ↓MIP-1β, ↓ KC, ↑PGE2 y LTB4 (5h), ↓ PGE2 y LTB4 (24h)	[125, 175]
Inflamación Broncoalveolar	↑Inflamación *Lavado Broncoalveolar:* ↑Linfocitos ↑Eosinófilos	*Lavado Broncoalveolar:* ↑IL-4 ↑IL-5 ↑Eotaxina ↑IgE	[207]
Pancreatitis inducida por Caerulina	↓Inflamación *Páncreas:* ↓Reclut. Neutrófilos ↓Act. Neutrófilos	*Páncreas:* ↓IL-6 ↓MCP-1 ↓MIP-2	[196]
Infección por *L. monocytogenes*	↑ Letalidad ↑ cargas bacterianas: hígado y bazo	Suero: ↔TNFα , ↑IL-12(p70) , ↑IL-27	[125]

Infección por *T. gondii*	↑Susceptibilidad ↑% de cel. peritoneales infectadas Bazo: ↔ NK y Células T	Suero: ↓ IFNγ, ↑ IL-12, ↔ IL-6, ↓ MCP-1	[207]
Colitis inducida por Sulfato Dextrano de Sodio	*Leve:* ↓Inflamación ↓% perdida peso *Aguda:* ↑tumores ↑Inflamación	*Leve:* Colon: ↓G-CSF, ↓GM-CSF, ↔ IP-10, ↓ KC, ↑MCP-1, ↓MIP-1α, ↓IL-1β, ↓ TNFα, ↓ IL-6, ↓ IL-1α, ↓ IL-13, ↓ IL-17, ↓ IL-12 (p70), ↓ IFNγ *Aguda:* Colon: ↑TNFα, ↑ KC, ↑ IFNγ, ↑ IL-6, ↑ IL-18, ↑ MCP-1	[87, 101]
Sobredosis de APAP DAMPs	↓Inflamación *Intraperitoneal* ↓Reclut. Neutrófilos ↓Reclut. Macrófagos ↓Reclut. NKT	Suero: ↓IL-1β, ↓ IL-6, ↓ IL-1α, ↓ IL-10	

La inflamación estéril es ampliamente dependiente de la IL-1α y su acción no puede ser substituida por IL-1β o cualquier otra citoquina [15, 41, 157]. El reclutamiento de neutrófilos al peritoneo en respuesta a DAMPs es de hecho dependiente, al menos en parte, de ésta citoquina [31], y se ha demostrado que tanto IL-1α como IL-1β son necesarias para el reclutamiento de diferentes células mieloides [157]. La también IL-1β regula la inflamación estéril, promoviendo el reclutamiento de neutrófilos y monocitos al foco inflamatorio y la inducción en la producción de nuevos mediadores pro-inflamatorios [50, 203]. Nuestro trabajo demuestra, que tras la activación de Cot/tpl-2, ésta media la producción de las citoquinas IL-1α y IL-1β, tanto *in vivo* como *in vitro*. Mientras que la deficiencia en Cot/tpl-2 en ratones APC KO resulta en una reducción de los niveles de IL-10 en suero [173], Cot/tpl-2 bloquea la producción de IL-10 tras el tratamiento con LPS y CpG-DNA [177]. Aunque se ha demostrado el papel protector de la IL-10 en el tratamiento con APAP [21], queda por determinar si el incremento en los niveles de esta citoquina observado por la deficiencia Cot/tpl-2 es la causa al menos en parte, de la menor lesión hepática tras la sobredosis de APAP en ratones.

Estudios *in vivo* con ratones Cot/tpl-2 KO en el modelo de inyección i.p. con Zymosan muestran una reducción en los niveles de diferentes citoquinas como TNFα, IL-1β e IL-6, además de un menor reclutamiento de leucocitos al foco inflamatorio [175]. Nuestros resultados demuestran que de igual forma, la deficiencia de Cot/tpl-2 reduce significativamente el número total de neutrófilos y macrófagos en la cavidad peritoneal tras la inyección de Wt o Cot/tpl-2 KO DAMPs. Además Cot/tpl-2 participa en la capacidad oxidativa de los leucocitos reclutados en el peritoneo en estado basal así como tras su estimulación con PMA.

Conclusiones

Las conclusiones más relevantes de esta Tesis Doctoral han sido:

1. Cot/tpl-2, mediante su capacidad para activar Erk1/2, promueve la traducción Cap-dependiente de los 5´ TOP mRNAs y de mensajeros de los mediadores inflamatorios. Además Cot/tpl-2 media la estabilización de estos mRNAs en macrófagos estimulados con LPS.

2. Cot/tpl-2 media la activación de Erk1/2 a través del eje IKKβ--p105-NFκB1--Cot/tpl-2--MKK1/2--Erk1/2 en macrófagos, tras la estimulación de éstos con DAMPs y APN.

3. La actividad quinasa de Cot/tpl-2 modula la inflamación estéril producida tras una sobredosis de APAP, mediante el reclutamiento de leucocitos al foco y la producción de mediadores inflamatorios.

4. Cot/tpl-2 modula la polarización de los macrófagos hacia un fenotipo M1 pro-inflamatorio tras la estimulación con DAMPs y APN.

Bibliografía

1. **Ajibade, A.A., H.Y. Wang, and R.F. Wang**. 2013. Cell type-specific function of TAK1 in innate immune signaling. Trends Immunol **34**(7): 307-316.

2. **Alder, M.N., I.B. Rogozin, L.M. Iyer, G.V. Glazko, M.D. Cooper, and Z. Pancer**. 2005. Diversity and function of adaptive immune receptors in a jawless vertebrate. Science **310**(5756): 1970-1973.

3. **Anjum, R. and J. Blenis**. 2008. The RSK family of kinases: emerging roles in cellular signalling. Nat Rev Mol Cell Biol **9**(10): 747-758.

4. **Antoniades, C., A.S. Antonopoulos, D. Tousoulis, and C. Stefanadis**. 2009. Adiponectin: from obesity to cardiovascular disease. Obes Rev **10**(3): 269-279.

5. **Antoniades, C.G., A. Quaglia, L.S. Taams, R.R. Mitry, M. Hussain, R. Abeles, L.A. Possamai, M. Bruce, M. McPhail, C. Starling, B. Wagner, A. Barnardo, S. Pomplun, G. Auzinger, W. Bernal, N. Heaton, D. Vergani, M.R. Thursz, and J. Wendon**. 2012. Source and characterization of hepatic macrophages in acetaminophen-induced acute liver failure in humans. Hepatology **56**(2): 735-746.

6. **Aoki, M., F. Hamada, T. Sugimoto, S. Sumida, T. Akiyama, and K. Toyoshima**. 1993. The human cot proto-oncogene encodes two protein serine/threonine kinases with different transforming activities by alternative initiation of translation. J Biol Chem **268**(30): 22723-22732.

7. **Arita, Y., S. Kihara, N. Ouchi, M. Takahashi, K. Maeda, J. Miyagawa, K. Hotta, I. Shimomura, T. Nakamura, K. Miyaoka, H. Kuriyama, M. Nishida, S. Yamashita, K. Okubo, K. Matsubara, M. Muraguchi, Y. Ohmoto, T. Funahashi, and Y. Matsuzawa**. 1999. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. Biochem Biophys Res Commun **257**(1): 79-83.

8. **Babelova, A., K. Moreth, W. Tsalastra-Greul, J. Zeng-Brouwers, O. Eickelberg, M.F. Young, P. Bruckner, J. Pfeilschifter, R.M. Schaefer, H.J. Grone, and L. Schaefer**. 2009. Biglycan, a danger signal that activates the NLRP3 inflammasome via toll-like and P2X receptors. J Biol Chem **284**(36): 24035-24048.

9. **Ballester, A., A. Velasco, R. Tobena, and S. Alemany**. 1998. Cot kinase activates tumor necrosis factor-alpha gene expression in a cyclosporin A-resistant manner. J Biol Chem **273**(23): 14099-14106.

10. **Bandow, K., J. Kusuyama, M. Shamoto, K. Kakimoto, T. Ohnishi, and T. Matsuguchi**. 2012. LPS-induced chemokine expression in both MyD88-dependent and -independent manners is regulated by Cot/Tpl2-ERK axis in macrophages. FEBS Lett **586**(10): 1540-1546.

11. **Banerjee, A., R. Gugasyan, M. McMahon, and S. Gerondakis**. 2006. Diverse Toll-like receptors utilize Tpl2 to activate extracellular signal-regulated kinase (ERK) in hemopoietic cells. Proc Natl Acad Sci U S A **103**(9): 3274-3279.

12. **Basu, S., R.J. Binder, T. Ramalingam, and P.K. Srivastava**. 2001. CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. Immunity **14**(3): 303-313.

13. **Beinke, S., J. Deka, V. Lang, M.P. Belich, P.A. Walker, S. Howell, S.J. Smerdon, S.J. Gamblin, and S.C. Ley**. 2003. NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity. Mol Cell Biol **23**(14): 4739-4752.

14. **Belich, M.P., A. Salmeron, L.H. Johnston, and S.C. Ley**. 1999. TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105. Nature **397**(6717): 363-368.

15. **Berda-Haddad, Y., S. Robert, P. Salers, L. Zekraoui, C. Farnarier, C.A. Dinarello, F. Dignat-George, and G. Kaplanski**. 2011. Sterile inflammation of endothelial cell-derived apoptotic bodies is mediated by interleukin-1alpha. Proc Natl Acad Sci U S A **108**(51): 20684-20689.

16. **Beretta, L., A.C. Gingras, Y.V. Svitkin, M.N. Hall, and N. Sonenberg**. 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J **15**(3): 658-664.

17. **Berg, A.H., T.P. Combs, and P.E. Scherer**. 2002. ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. Trends Endocrinol Metab **13**(2): 84-89.

18. **Blazer, D.G. and L.T. Wu**. 2009. Nonprescription use of pain relievers by middle-aged and elderly community-living adults: National Survey on Drug Use and Health. J Am Geriatr Soc **57**(7): 1252-1257.

19. **Blazka, M.E., J.L. Wilmer, S.D. Holladay, R.E. Wilson, and M.I. Luster**. 1995. Role of proinflammatory cytokines in acetaminophen hepatotoxicity. Toxicol Appl Pharmacol **133**(1): 43-52.

20. **Bogdan, C.** 2001. Nitric oxide and the immune response. Nat Immunol **2**(10): 907-916.

21. **Bourdi, M., Y. Masubuchi, T.P. Reilly, H.R. Amouzadeh, J.L. Martin, J.W. George, A.G. Shah, and L.R. Pohl**. 2002. Protection against acetaminophen-induced liver injury and lethality by interleukin 10: role of inducible nitric oxide synthase. Hepatology **35**(2): 289-298.

22. **Bours, M.J., E.L. Swennen, F. Di Virgilio, B.N. Cronstein, and P.C. Dagnelie**. 2006. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther **112**(2): 358-404.

23. **Caivano, M., C. Rodriguez, P. Cohen, and S. Alemany**. 2003. 15-Deoxy-Delta12,14-prostaglandin J2 regulates endogenous Cot MAPK kinase kinase 1 activity induced by lipopolysaccharide. J Biol Chem **278**(52): 52124-52130.

24. **Cayrol, C. and J.P. Girard**. 2009. The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. Proc Natl Acad Sci U S A **106**(22): 9021-9026.

25. **Ceci, J.D., C.P. Patriotis, C. Tsatsanis, A.M. Makris, R. Kovatch, D.A. Swing, N.A. Jenkins, P.N. Tsichlis, and N.G. Copeland**. 1997. Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. Genes Dev **11**(6): 688-700.

26. **Cohen, P.** 2009. Targeting protein kinases for the development of anti-inflammatory drugs. Curr Opin Cell Biol **21**(2): 317-324.

27. **Cusack, K., H. Allen, A. Bischoff, A. Clabbers, R. Dixon, S. Fix-Stenzel, M. Friedman, Y. Gaumont, D. George, T. Gordon, P. Grongsaard, B. Janssen, Y. Jia, M. Moskey, C. Quinn, A. Salmeron, C. Thomas, G. Wallace, N. Wishart, and Z. Yu**. 2009. Identification of a selective thieno[2,3-c]pyridine inhibitor of COT kinase and TNF-alpha production. Bioorg Med Chem Lett **19**(6): 1722-1725.

28. **Chakraborty, R., K.H. Bhatt, and A. Sodhi**. 2013. High mobility group box 1 protein synergizes with lipopolysaccharide and peptidoglycan for nitric oxide production in mouse peritoneal macrophages in vitro. Mol Immunol **54**(1): 48-57.

29. **Chandran, M., S.A. Phillips, T. Ciaraldi, and R.R. Henry**. 2003. Adiponectin: more than just another fat cell hormone? Diabetes Care **26**(8): 2442-2450.

30. **Chawla, A.** 2010. Control of macrophage activation and function by PPARs. Circ Res **106**(10): 1559-1569.

31. **Chen, C.J., H. Kono, D. Golenbock, G. Reed, S. Akira, and K.L. Rock**. 2007. Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat Med **13**(7): 851-856.

32. **Chen, G.Y. and G. Nunez**. 2010. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol **10**(12): 826-837.

33. **Chen, G.Y., J. Tang, P. Zheng, and Y. Liu**. 2009. CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. Science **323**(5922): 1722-1725.

34. **Cheng, X., E.J. Folco, K. Shimizu, and P. Libby**. 2012. Adiponectin induces pro-inflammatory programs in human macrophages and CD4+ T cells. J Biol Chem **287**(44): 36896-36904.

35. **Chiariello, M., M.J. Marinissen, and J.S. Gutkind**. 2000. Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. Mol Cell Biol **20**(5): 1747-1758.

36. **Das, S., J. Cho, I. Lambertz, M.A. Kelliher, A.G. Eliopoulos, K. Du, and P.N. Tsichlis**. 2005. Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. J Biol Chem **280**(25): 23748-23757.

37. **Decicco-Skinner, K.L., E.L. Trovato, J.K. Simmons, P.K. Lepage, and J.S. Wiest**. 2011. Loss of tumor progression locus 2 (tpl2) enhances tumorigenesis and inflammation in two-stage skin carcinogenesis. Oncogene **30**(4): 389-397.

38. **Diefenbach, A., H. Schindler, N. Donhauser, E. Lorenz, T. Laskay, J. MacMicking, M. Rollinghoff, I. Gresser, and C. Bogdan**. 1998. Type 1 interferon (IFNalpha/beta) and type 2 nitric oxide synthase regulate the innate immune response to a protozoan parasite. Immunity **8**(1): 77-87.

39. **Dumitru, C.D., J.D. Ceci, C. Tsatsanis, D. Kontoyiannis, K. Stamatakis, J.H. Lin, C. Patriotis, N.A. Jenkins, N.G. Copeland, G. Kollias, and P.N. Tsichlis**. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell **103**(7): 1071-1083.

40. **Ehrstrom, M., E. Naslund, J. Ma, A.L. Kirchgessner, and P.M. Hellstrom**. 2003. Physiological regulation and NO-dependent inhibition of migrating myoelectric complex in the rat small bowel by OXA. Am J Physiol Gastrointest Liver Physiol **285**(4): G688-695.

41. **Eigenbrod, T., J.H. Park, J. Harder, Y. Iwakura, and G. Nunez**. 2008. Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. J Immunol **181**(12): 8194-8198.

42. **Eliopoulos, A.G., C.C. Wang, C.D. Dumitru, and P.N. Tsichlis**. 2003. Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40. EMBO J **22**(15): 3855-3864.

43. **Eliopoulos, A.G., C.D. Dumitru, C.C. Wang, J. Cho, and P.N. Tsichlis**. 2002. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J **21**(18): 4831-4840.

44. **Erny, K.M., J. Peli, J.F. Lambert, V. Muller, and H. Diggelmann**. 1996. Involvement of the Tpl-2/cot oncogene in MMTV tumorigenesis. Oncogene **13**(9): 2015-2020.

45. **Fairweather, D. and D. Cihakova**. 2009. Alternatively activated macrophages in infection and autoimmunity. J Autoimmun **33**(3-4): 222-230.

46. **Fasshauer, M., S. Kralisch, M. Klier, U. Lossner, M. Bluher, J. Klein, and R. Paschke**. 2003. Adiponectin gene expression and secretion is inhibited by interleukin-6 in 3T3-L1 adipocytes. Biochem Biophys Res Commun **301**(4): 1045-1050.

47. **Folco, E.J., V.Z. Rocha, M. Lopez-Ilasaca, and P. Libby**. 2009. Adiponectin inhibits pro-inflammatory signaling in human macrophages independent of interleukin-10. J Biol Chem **284**(38): 25569-25575.

48. **Foster, J.G., M.D. Blunt, E. Carter, and S.G. Ward**. 2012. Inhibition of PI3K signaling spurs new therapeutic opportunities in inflammatory/autoimmune diseases and hematological malignancies. Pharmacol Rev **64**(4): 1027-1054.

49. **Fukao, T. and S. Koyasu**. 2003. PI3K and negative regulation of TLR signaling. Trends Immunol **24**(7): 358-363.

50. **Gabay, C., C. Lamacchia, and G. Palmer**. 2010. IL-1 pathways in inflammation and human diseases. Nat Rev Rheumatol **6**(4): 232-241.

51. **Gaestel, M., A. Kotlyarov, and M. Kracht**. 2009. Targeting innate immunity protein kinase signalling in inflammation. Nat Rev Drug Discov **8**(6): 480-499.

52. **Gaestel, M., A. Mengel, U. Bothe, and K. Asadullah**. 2007. Protein kinases as small molecule inhibitor targets in inflammation. Curr Med Chem **14**(21): 2214-2234.

53. **Gandara, M.L., P. Lopez, R. Hernando, J.G. Castano, and S. Alemany**. 2003. The COOH-terminal domain of wild-type Cot regulates its stability and kinase specific activity. Mol Cell Biol **23**(20): 7377-7390.

54. **Gantke, T., S. Sriskantharajah, and S.C. Ley**. 2011. Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase. Cell Res **21**(1): 131-145.

55. **Gantner, B.N., R.M. Simmons, S.J. Canavera, S. Akira, and D.M. Underhill**. 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. J Exp Med **197**(9): 1107-1117.

56. **Garcia-Martinez, J.M. and D.R. Alessi**. 2008. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J **416**(3): 375-385.

57. **Gil-Campos, M., R.R. Canete, and A. Gil**. 2004. Adiponectin, the missing link in insulin resistance and obesity. Clin Nutr **23**(5): 963-974.

58. **Gordon, S.** 2003. Alternative activation of macrophages. Nat Rev Immunol **3**(1): 23-35.

59. **Gordon, S. and P.R. Taylor**. 2005. Monocyte and macrophage heterogeneity. Nat Rev Immunol **5**(12): 953-964.

60. **Green, N., Y. Hu, K. Janz, H.Q. Li, N. Kaila, S. Guler, J. Thomason, D. Joseph-McCarthy, S.Y. Tam, R. Hotchandani, J. Wu, A. Huang, Q. Wang, L. Leung, J. Pelker, S. Marusic, S. Hsu, J.B. Telliez, J.P. Hall, J.W. Cuozzo, and L.L. Lin**. 2007. Inhibitors of tumor progression loci-2 (Tpl2) kinase and tumor necrosis factor alpha (TNF-alpha) production: selectivity and in vivo antiinflammatory activity of novel 8-substituted-4-anilino-6-aminoquinoline-3-carbonitriles. J Med Chem **50**(19): 4728-4745.

61. **Guertin, D.A., D.M. Stevens, C.C. Thoreen, A.A. Burds, N.Y. Kalaany, J. Moffat, M. Brown, K.J. Fitzgerald, and D.M. Sabatini**. 2006. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell **11**(6): 859-871.

62. **Gunawan, B.K., Z.X. Liu, D. Han, N. Hanawa, W.A. Gaarde, and N. Kaplowitz**. 2006. c-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. Gastroenterology **131**(1): 165-178.

63. **Hacker, H., P.H. Tseng, and M. Karin**. 2011. Expanding TRAF function: TRAF3 as a tri-faced immune regulator. Nat Rev Immunol **11**(7): 457-468.

64. **Hattori, Y., S. Hattori, and K. Kasai**. 2006. Globular adiponectin activates nuclear factor-kappaB in vascular endothelial cells, which in turn induces expression of proinflammatory and adhesion molecule genes. Diabetes Care **29**(1): 139-141.

65. **Hatziapostolou, M., C. Polytarchou, D. Panutsopulos, L. Covic, and P.N. Tsichlis**. 2008. Proteinase-activated receptor-1-triggered activation of tumor progression locus-2 promotes actin cytoskeleton reorganization and cell migration. Cancer Res **68**(6): 1851-1861.

66. **Heissmeyer, V., D. Krappmann, E.N. Hatada, and C. Scheidereit**. 2001. Shared pathways of IkappaB kinase-induced SCF(betaTrCP)-mediated ubiquitination and degradation for the NF-kappaB precursor p105 and IkappaBalpha. Mol Cell Biol **21**(4): 1024-1035.

67. **Hendrix, C., J. Anne, B. Joris, A. Van Aerschot, and P. Herdewijn**. 1996. Selection of hammerhead ribozymes for optimum cleavage of interleukin 6 mRNA. Biochem J **314 (Pt 2)**: 655-661.

68. **Holt, M.P. and C. Ju**. 2006. Mechanisms of drug-induced liver injury. AAPS J **8**(1): E48-54.

69. **Holt, M.P., L. Cheng, and C. Ju**. 2008. Identification and characterization of infiltrating macrophages in acetaminophen-induced liver injury. J Leukoc Biol **84**(6): 1410-1421.

70. **Hoque, R., A. Farooq, and W.Z. Mehal**. 2013. Sterile inflammation in the liver and pancreas. J Gastroenterol Hepatol **28 Suppl 1**: 61-67.

71. **Hotta, K., T. Funahashi, Y. Arita, M. Takahashi, M. Matsuda, Y. Okamoto, H. Iwahashi, H. Kuriyama, N. Ouchi, K. Maeda, M. Nishida, S. Kihara, N. Sakai, T. Nakajima, K. Hasegawa, M. Muraguchi, Y. Ohmoto, T. Nakamura, S. Yamashita, T. Hanafusa, and Y. Matsuzawa**. 2000. Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. Arterioscler Thromb Vasc Biol **20**(6): 1595-1599.

72. **Hu, E., P. Liang, and B.M. Spiegelman**. 1996. AdipoQ is a novel adipose-specific gene dysregulated in obesity. J Biol Chem **271**(18): 10697-10703.

73. **Hug, C., J. Wang, N.S. Ahmad, J.S. Bogan, T.S. Tsao, and H.F. Lodish**. 2004. T-cadherin is a receptor for hexameric and high-molecular-weight forms of Acrp30/adiponectin. Proc Natl Acad Sci U S A **101**(28): 10308-10313.

74. **Imaeda, A.B., A. Watanabe, M.A. Sohail, S. Mahmood, M. Mohamadnejad, F.S. Sutterwala, R.A. Flavell, and W.Z. Mehal**. 2009. Acetaminophen-induced hepatotoxicity in mice is dependent on Tlr9 and the Nalp3 inflammasome. J Clin Invest **119**(2): 305-314.

75. **Iwasaki, A. and R. Medzhitov**. 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol **5**(10): 987-995.

76. **Iwashima, Y., T. Katsuya, K. Ishikawa, N. Ouchi, M. Ohishi, K. Sugimoto, Y. Fu, M. Motone, K. Yamamoto, A. Matsuo, K. Ohashi, S. Kihara, T. Funahashi, H. Rakugi, Y.**

Matsuzawa, and T. Ogihara. 2004. Hypoadiponectinemia is an independent risk factor for hypertension. Hypertension **43**(6): 1318-1323.

77. **Jaeschke, H., J.S. Gujral, and M.L. Bajt**. 2004. Apoptosis and necrosis in liver disease. Liver Int **24**(2): 85-89.

78. **Jiang, D., J. Liang, J. Fan, S. Yu, S. Chen, Y. Luo, G.D. Prestwich, M.M. Mascarenhas, H.G. Garg, D.A. Quinn, R.J. Homer, D.R. Goldstein, R. Bucala, P.J. Lee, R. Medzhitov, and P.W. Noble**. 2005. Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med **11**(11): 1173-1179.

79. **Jones, D.P., J.J. Lemasters, D. Han, U.A. Boelsterli, and N. Kaplowitz**. 2010. Mechanisms of pathogenesis in drug hepatotoxicity putting the stress on mitochondria. Mol Interv **10**(2): 98-111.

80. **Jung, M.Y., H.S. Kim, H.J. Hong, B.S. Youn, and T.S. Kim**. 2012. Adiponectin induces dendritic cell activation via PLCgamma/JNK/NF-kappaB pathways, leading to Th1 and Th17 polarization. J Immunol **188**(6): 2592-2601.

81. **Kadowaki, T. and T. Yamauchi**. 2005. Adiponectin and adiponectin receptors. Endocr Rev **26**(3): 439-451.

82. **Kaila, N., N. Green, H.Q. Li, Y. Hu, K. Janz, L.K. Gavrin, J. Thomason, S. Tam, D. Powell, J. Cuozzo, J.P. Hall, J.B. Telliez, S. Hsu, C. Nickerson-Nutter, Q. Wang, and L.L. Lin**. 2007. Identification of a novel class of selective Tpl2 kinase inhibitors: 4-Alkylamino-[1,7]naphthyridine-3-carbonitriles. Bioorg Med Chem **15**(19): 6425-6442.

83. **Kaiser, F., D. Cook, S. Papoutsopoulou, R. Rajsbaum, X. Wu, H.T. Yang, S. Grant, P. Ricciardi-Castagnoli, P.N. Tsichlis, S.C. Ley, and A. O'Garra**. 2009. TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. J Exp Med **206**(9): 1863-1871.

84. **Kawai, T. and S. Akira**. 2005. Toll-like receptor downstream signaling. Arthritis Res Ther **7**(1): 12-19.

85. **Kawai, T. and S. Akira**. 2006. TLR signaling. Cell Death Differ **13**(5): 816-825.

86. **Kisilevsky, R. and S.P. Tam**. 2003. Macrophage cholesterol efflux and the active domains of serum amyloid A 2.1. J Lipid Res **44**(12): 2257-2269.

87. **Koliaraki, V., M. Roulis, and G. Kollias**. 2012. Tpl2 regulates intestinal myofibroblast HGF release to suppress colitis-associated tumorigenesis. J Clin Invest **122**(11): 4231-4242.

88. **Kondo, T., T. Kawai, and S. Akira**. 2012. Dissecting negative regulation of Toll-like receptor signaling. Trends Immunol **33**(9): 449-458.

89. **Kono, D.H., M.K. Haraldsson, B.R. Lawson, K.M. Pollard, Y.T. Koh, X. Du, C.N. Arnold, R. Baccala, G.J. Silverman, B.A. Beutler, and A.N. Theofilopoulos**. 2009. Endosomal TLR signaling is required for anti-nucleic acid and rheumatoid factor autoantibodies in lupus. Proc Natl Acad Sci U S A **106**(29): 12061-12066.

90. **Kono, H., D. Karmarkar, Y. Iwakura, and K.L. Rock**. 2010. Identification of the cellular sensor that stimulates the inflammatory response to sterile cell death. J Immunol **184**(8): 4470-4478.

91. **Kono, H., C.J. Chen, F. Ontiveros, and K.L. Rock**. 2010. Uric acid promotes an acute inflammatory response to sterile cell death in mice. J Clin Invest **120**(6): 1939-1949.

92. **Konter, J.M., J.L. Parker, E. Baez, S.Z. Li, B. Ranscht, M. Denzel, F.F. Little, K. Nakamura, N. Ouchi, A. Fine, K. Walsh, and R.S. Summer**. 2012. Adiponectin attenuates lipopolysaccharide-induced acute lung injury through suppression of endothelial cell activation. J Immunol **188**(2): 854-863.

93. **Kontoyiannis, D., G. Boulougouris, M. Manoloukos, M. Armaka, M. Apostolaki, T. Pizarro, A. Kotlyarov, I. Forster, R. Flavell, M. Gaestel, P. Tsichlis, F. Cominelli, and G. Kollias**. 2002. Genetic dissection of the cellular pathways and signaling mechanisms in modeled tumor necrosis factor-induced Crohn's-like inflammatory bowel disease. J Exp Med **196**(12): 1563-1574.

94. **Kredel, L.I., A. Batra, T. Stroh, A.A. Kuhl, M. Zeitz, U. Erben, and B. Siegmund**. 2013. Adipokines from local fat cells shape the macrophage compartment of the creeping fat in Crohn's disease. Gut **62**(6): 852-862.

95. **Krishna, M. and H. Narang**. 2008. The complexity of mitogen-activated protein kinases (MAPKs) made simple. Cell Mol Life Sci **65**(22): 3525-3544.

96. **Kubota, N., Y. Terauchi, T. Yamauchi, T. Kubota, M. Moroi, J. Matsui, K. Eto, T. Yamashita, J. Kamon, H. Satoh, W. Yano, P. Froguel, R. Nagai, S. Kimura, T. Kadowaki, and T. Noda**. 2002. Disruption of adiponectin causes insulin resistance and neointimal formation. J Biol Chem **277**(29): 25863-25866.

97. **Kubota, N., Y. Terauchi, T. Kubota, H. Kumagai, S. Itoh, H. Satoh, W. Yano, H. Ogata, K. Tokuyama, I. Takamoto, T. Mineyama, M. Ishikawa, M. Moroi, K. Sugi, T. Yamauchi, K. Ueki, K. Tobe, T. Noda, R. Nagai, and T. Kadowaki**. 2006. Pioglitazone ameliorates insulin resistance and diabetes by both adiponectin-dependent and -independent pathways. J Biol Chem **281**(13): 8748-8755.

98. **Kumada, M., S. Kihara, S. Sumitsuji, T. Kawamoto, S. Matsumoto, N. Ouchi, Y. Arita, Y. Okamoto, I. Shimomura, H. Hiraoka, T. Nakamura, T. Funahashi, Y. Matsuzawa, and C.A.D.S.G.C.a.d. Osaka**. 2003. Association of hypoadiponectinemia with coronary artery disease in men. Arterioscler Thromb Vasc Biol **23**(1): 85-89.

99. **Kyrmizi, I., M. Ioannou, M. Hatziapostolou, P.N. Tsichlis, D.T. Boumpas, and I. Tassiulas**. 2013. Tpl2 kinase regulates FcgammaR signaling and immune thrombocytopenia in mice. J Leukoc Biol.

100. **Lang, V., A. Symons, S.J. Watton, J. Janzen, Y. Soneji, S. Beinke, S. Howell, and S.C. Ley**. 2004. ABIN-2 forms a ternary complex with TPL-2 and NF-kappa B1 p105 and is essential for TPL-2 protein stability. Mol Cell Biol **24**(12): 5235-5248.

101. **Lawrenz, M., A. Visekruna, A. Kuhl, N. Schmidt, S.H. Kaufmann, and U. Steinhoff**. 2012. Genetic and pharmacological targeting of TPL-2 kinase ameliorates experimental colitis: a potential target for the treatment of Crohn's disease? Mucosal Immunol **5**(2): 129-139.

102. **Li, F.Y., K.S. Lam, and A. Xu**. 2012. Therapeutic perspectives for adiponectin: an update. Curr Med Chem **19**(32): 5513-5523.

103. **Li, L. and L.L. Wu**. 2012. Adiponectin and interleukin-6 in inflammation-associated disease. Vitam Horm **90**: 375-395.

104. **Li, Y.L., C. Torchet, J. Vergne, and M.C. Maurel**. 2007. A cis and trans adenine-dependent hairpin ribozyme against Tpl-2 target. Biochimie **89**(10): 1257-1263.

105. **Li, Y.L., J. Vergne, C. Torchet, and M.C. Maurel**. 2009. In vitro selection of adenine-dependent ribozyme against Tpl2/Cot oncogene. FEBS J **276**(1): 303-314.

106. **Lin, Q., M. Li, D. Fang, J. Fang, and S.B. Su**. 2011. The essential roles of Toll-like receptor signaling pathways in sterile inflammatory diseases. Int Immunopharmacol **11**(10): 1422-1432.

107. **Lin, S.C., Y.C. Lo, and H. Wu**. 2010. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. Nature **465**(7300): 885-890.

108. **Loniewski, K.J., S. Patial, and N. Parameswaran**. 2007. Sensitivity of TLR4- and -7-induced NF kappa B1 p105-TPL2-ERK pathway to TNF-receptor-associated-factor-6 revealed by RNAi in mouse macrophages. Mol Immunol **44**(15): 3715-3723.

109. **Lopez-Pelaez, M., I. Soria-Castro, L. Bosca, M. Fernandez, and S. Alemany**. 2011. Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. Eur J Immunol **41**(6): 1733-1741.

110. **Lowenstein, C.J. and E. Padalko**. 2004. iNOS (NOS2) at a glance. J Cell Sci **117**(Pt 14): 2865-2867.

111. **Luciano, B.S., S. Hsu, P.L. Channavajhala, L.L. Lin, and J.W. Cuozzo**. 2004. Phosphorylation of threonine 290 in the activation loop of Tpl2/Cot is necessary but not sufficient for kinase activity. J Biol Chem **279**(50): 52117-52123.

112. **Ma, J., T. Chen, J. Mandelin, A. Ceponis, N.E. Miller, M. Hukkanen, G.F. Ma, and Y.T. Konttinen**. 2003. Regulation of macrophage activation. Cell Mol Life Sci **60**(11): 2334-2346.

113. **Ma, L., Z. Chen, H. Erdjument-Bromage, P. Tempst, and P.P. Pandolfi**. 2005. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell **121**(2): 179-193.

114. **Ma, X.M. and J. Blenis**. 2009. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol **10**(5): 307-318.

115. **MacKenzie, K.F., M.W. Van Den Bosch, S. Naqvi, S.E. Elcombe, V.A. McGuire, A.D. Reith, P.J. Blackshear, J.L. Dean, and J.S. Arthur**. 2013. MSK1 and MSK2 inhibit lipopolysaccharide-induced prostaglandin production via an interleukin-10 feedback loop. Mol Cell Biol **33**(7): 1456-1467.

116. **Maeda, K., K. Okubo, I. Shimomura, T. Funahashi, Y. Matsuzawa, and K. Matsubara**. 1996. cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (AdiPose Most abundant Gene transcript 1). Biochem Biophys Res Commun **221**(2): 286-289.

117. **Malhi, H., G.J. Gores, and J.J. Lemasters**. 2006. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology **43**(2 Suppl 1): S31-44.

118. **Mao, X., C.K. Kikani, R.A. Riojas, P. Langlais, L. Wang, F.J. Ramos, Q. Fang, C.Y. Christ-Roberts, J.Y. Hong, R.Y. Kim, F. Liu, and L.Q. Dong**. 2006. APPL1 binds to adiponectin receptors and mediates adiponectin signalling and function. Nat Cell Biol **8**(5): 516-523.

119. **Martinez, F.O.** 2011. Regulators of macrophage activation. Eur J Immunol **41**(6): 1531-1534.

120. **Masek, T., L. Valasek, and M. Pospisek**. 2011. Polysome analysis and RNA purification from sucrose gradients. Methods Mol Biol **703**: 293-309.

121. **Medzhitov, R.** 2008. Origin and physiological roles of inflammation. Nature **454**(7203): 428-435.

122. **Medzhitov, R. and C.A. Janeway, Jr.** 1997. Innate immunity: impact on the adaptive immune response. Curr Opin Immunol **9**(1): 4-9.

123. **Meek, R.L., S. Urieli-Shoval, and E.P. Benditt**. 1994. Expression of apolipoprotein serum amyloid A mRNA in human atherosclerotic lesions and cultured vascular cells: implications for serum amyloid A function. Proc Natl Acad Sci U S A **91**(8): 3186-3190.

124. **Meyers, L.L., W.P. Beierschmitt, E.A. Khairallah, and S.D. Cohen**. 1988. Acetaminophen-induced inhibition of hepatic mitochondrial respiration in mice. Toxicol Appl Pharmacol **93**(3): 378-387.

125. **Mielke, L.A., K.L. Elkins, L. Wei, R. Starr, P.N. Tsichlis, J.J. O'Shea, and W.T. Watford**. 2009. Tumor progression locus 2 (Map3k8) is critical for host defense against Listeria monocytogenes and IL-1 beta production. J Immunol **183**(12): 7984-7993.

126. **Mitchell, J.R., D.J. Jollow, W.Z. Potter, J.R. Gillette, and B.B. Brodie**. 1973. Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. J Pharmacol Exp Ther **187**(1): 211-217.

127. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi**. 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell **54**(6): 903-913.

128. **Miyoshi, J., T. Higashi, H. Mukai, T. Ohuchi, and T. Kakunaga**. 1991. Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. Mol Cell Biol **11**(8): 4088-4096.

129. **Moon, M.L., L.K. McNeil, and G.G. Freund**. 2011. Macrophages make me sick: how macrophage activation states influence sickness behavior. Psychoneuroendocrinology **36**(10): 1431-1440.

130. **Moresco, E.M., D. LaVine, and B. Beutler**. 2011. Toll-like receptors. Curr Biol **21**(13): R488-493.

131. **Motoshima, H., X. Wu, K. Mahadev, and B.J. Goldstein**. 2004. Adiponectin suppresses proliferation and superoxide generation and enhances eNOS activity in endothelial cells treated with oxidized LDL. Biochem Biophys Res Commun **315**(2): 264-271.

132. **Motoshima, H., X. Wu, M.K. Sinha, V.E. Hardy, E.L. Rosato, D.J. Barbot, F.E. Rosato, and B.J. Goldstein**. 2002. Differential regulation of adiponectin secretion from cultured human omental and subcutaneous adipocytes: effects of insulin and rosiglitazone. J Clin Endocrinol Metab **87**(12): 5662-5667.

133. **Moussion, C., N. Ortega, and J.P. Girard**. 2008. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS One **3**(10): e3331.

134. **Murray, P.J.** 2007. The JAK-STAT signaling pathway: input and output integration. J Immunol **178**(5): 2623-2629.

135. **Murray, P.J. and T.A. Wynn**. 2011. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol **11**(11): 723-737.

136. **Nakano, Y., T. Tobe, N.H. Choi-Miura, T. Mazda, and M. Tomita**. 1996. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. J Biochem **120**(4): 803-812.

137. **Nilsson, L., N. Binart, Y.M. Bohlooly, M. Bramnert, E. Egecioglu, J. Kindblom, P.A. Kelly, J.J. Kopchick, C.J. Ormandy, C. Ling, and H. Billig**. 2005. Prolactin and growth

hormone regulate adiponectin secretion and receptor expression in adipose tissue. Biochem Biophys Res Commun **331**(4): 1120-1126.

138. **O'Garra, A., F.J. Barrat, A.G. Castro, A. Vicari, and C. Hawrylowicz**. 2008. Strategies for use of IL-10 or its antagonists in human disease. Immunol Rev **223**: 114-131.

139. **Ohashi, K., J.L. Parker, N. Ouchi, A. Higuchi, J.A. Vita, N. Gokce, A.A. Pedersen, C. Kalthoff, S. Tullin, A. Sams, R. Summer, and K. Walsh**. 2010. Adiponectin promotes macrophage polarization toward an anti-inflammatory phenotype. J Biol Chem **285**(9): 6153-6160.

140. **Ohmori, Y. and T.A. Hamilton**. 2001. Requirement for STAT1 in LPS-induced gene expression in macrophages. J Leukoc Biol **69**(4): 598-604.

141. **Okamoto, Y., E.J. Folco, M. Minami, A.K. Wara, M.W. Feinberg, G.K. Sukhova, R.A. Colvin, S. Kihara, T. Funahashi, A.D. Luster, and P. Libby**. 2008. Adiponectin inhibits the production of CXC receptor 3 chemokine ligands in macrophages and reduces T-lymphocyte recruitment in atherogenesis. Circ Res **102**(2): 218-225.

142. **Ouchi, N., S. Kihara, Y. Arita, K. Maeda, H. Kuriyama, Y. Okamoto, K. Hotta, M. Nishida, M. Takahashi, T. Nakamura, S. Yamashita, T. Funahashi, and Y. Matsuzawa**. 1999. Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein adiponectin. Circulation **100**(25): 2473-2476.

143. **Ouchi, N., S. Kihara, Y. Arita, M. Nishida, A. Matsuyama, Y. Okamoto, M. Ishigami, H. Kuriyama, K. Kishida, H. Nishizawa, K. Hotta, M. Muraguchi, Y. Ohmoto, S. Yamashita, T. Funahashi, and Y. Matsuzawa**. 2001. Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. Circulation **103**(8): 1057-1063.

144. **Pajvani, U.B., X. Du, T.P. Combs, A.H. Berg, M.W. Rajala, T. Schulthess, J. Engel, M. Brownlee, and P.E. Scherer**. 2003. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. J Biol Chem **278**(11): 9073-9085.

145. **Palanisamy, V., A. Jakymiw, E.A. Van Tubergen, N.J. D'Silva, and K.L. Kirkwood**. 2012. Control of cytokine mRNA expression by RNA-binding proteins and microRNAs. J Dent Res **91**(7): 651-658.

146. **Panda, A., A. Arjona, E. Sapey, F. Bai, E. Fikrig, R.R. Montgomery, J.M. Lord, and A.C. Shaw**. 2009. Human innate immunosenescence: causes and consequences for immunity in old age. Trends Immunol **30**(7): 325-333.

147. **Papoutsopoulou, S., A. Symons, T. Tharmalingham, M.P. Belich, F. Kaiser, D. Kioussis, A. O'Garra, V. Tybulewicz, and S.C. Ley**. 2006. ABIN-2 is required for optimal activation of Erk MAP kinase in innate immune responses. Nat Immunol **7**(6): 606-615.

148. **Pasare, C. and R. Medzhitov**. 2004. Toll-like receptors and acquired immunity. Semin Immunol **16**(1): 23-26.

149. **Patriotis, C., A. Makris, S.E. Bear, and P.N. Tsichlis**. 1993. Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. Proc Natl Acad Sci U S A **90**(6): 2251-2255.

150. **Patriotis, C., A. Makris, J. Chernoff, and P.N. Tsichlis**. 1994. Tpl-2 acts in concert with Ras and Raf-1 to activate mitogen-activated protein kinase. Proc Natl Acad Sci U S A **91**(21): 9755-9759.

151. **Paust, S. and U.H. von Andrian**. 2011. Natural killer cell memory. Nat Immunol **12**(6): 500-508.

152. **Perugorria, M.J., L.B. Murphy, N. Fullard, J.B. Chakraborty, D. Vyrla, C.L. Wilson, F. Oakley, J. Mann, and D.A. Mann**. 2013. Tumor progression locus 2/Cot is required for activation of extracellular regulated kinase in liver injury and toll-like receptor-induced TIMP-1 gene transcription in hepatic stellate cells in mice. Hepatology **57**(3): 1238-1249.

153. **Piccinini, A.M. and K.S. Midwood**. 2010. DAMPening inflammation by modulating TLR signalling. Mediators Inflamm **2010**.

154. **Pichon, X., L.A. Wilson, M. Stoneley, A. Bastide, H.A. King, J. Somers, and A.E. Willis**. 2012. RNA binding protein/RNA element interactions and the control of translation. Curr Protein Pept Sci **13**(4): 294-304.

155. **Proud, C.G.** 2009. mTORC1 signalling and mRNA translation. Biochem Soc Trans **37**(Pt 1): 227-231.

156. **Quintana, F.J. and I.R. Cohen**. 2005. Heat shock proteins as endogenous adjuvants in sterile and septic inflammation. J Immunol **175**(5): 2777-2782.

157. **Rider, P., Y. Carmi, O. Guttman, A. Braiman, I. Cohen, E. Voronov, M.R. White, C.A. Dinarello, and R.N. Apte**. 2011. IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. J Immunol **187**(9): 4835-4843.

158. **Robinson, M.J., S. Beinke, A. Kouroumalis, P.N. Tsichlis, and S.C. Ley**. 2007. Phosphorylation of TPL-2 on serine 400 is essential for lipopolysaccharide activation of extracellular signal-regulated kinase in macrophages. Mol Cell Biol **27**(21): 7355-7364.

159. **Rodriguez, C., M. Pozo, E. Nieto, M. Fernandez, and S. Alemany**. 2006. TRAF6 and Src kinase activity regulates Cot activation by IL-1. Cell Signal **18**(9): 1376-1385.

160. **Rossi, A. and J. Lord**. 2013. Adiponectin Inhibits Neutrophil Phagocytosis of Escherichia coli by Inhibition of PKB and ERK 1/2 MAPK Signalling and Mac-1 Activation. PLoS One **8**(7): e69108.

161. **Rousseau, S., M. Papoutsopoulou, A. Symons, D. Cook, J.M. Lucocq, A.R. Prescott, A. O'Garra, S.C. Ley, and P. Cohen**. 2008. TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. J Cell Sci **121**(Pt 2): 149-154.

162. **Roux, P.P., B.A. Ballif, R. Anjum, S.P. Gygi, and J. Blenis**. 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A **101**(37): 13489-13494.

163. **Saijo, S., K. Nagata, Y. Nakano, T. Tobe, and Y. Kobayashi**. 2005. Inhibition by adiponectin of IL-8 production by human macrophages upon coculturing with late apoptotic cells. Biochem Biophys Res Commun **334**(4): 1180-1183.

164. **Salmenniemi, U., E. Ruotsalainen, J. Pihlajamaki, I. Vauhkonen, S. Kainulainen, K. Punnonen, E. Vanninen, and M. Laakso**. 2004. Multiple abnormalities in glucose and energy metabolism and coordinated changes in levels of adiponectin, cytokines, and adhesion molecules in subjects with metabolic syndrome. Circulation **110**(25): 3842-3848.

165. **Salmeron, A., T.B. Ahmad, G.W. Carlile, D. Pappin, R.P. Narsimhan, and S.C. Ley**. 1996. Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. EMBO J **15**(4): 817-826.

166. **Salmeron, A., J. Janzen, Y. Soneji, N. Bump, J. Kamens, H. Allen, and S.C. Ley**. 2001. Direct phosphorylation of NF-kappaB1 p105 by the IkappaB kinase complex on serine 927 is essential for signal-induced p105 proteolysis. J Biol Chem **276**(25): 22215-22222.

167. **Sato, C., Z. Yasukawa, N. Honda, T. Matsuda, and K. Kitajima**. 2001. Identification and adipocyte differentiation-dependent expression of the unique disialic acid residue in an adipose tissue-specific glycoprotein, adipo Q. J Biol Chem **276**(31): 28849-28856.

168. **Sato, S., H. Sanjo, K. Takeda, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, K. Matsumoto, O. Takeuchi, and S. Akira**. 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. Nat Immunol **6**(11): 1087-1095.

169. **Savinova, O.V., A. Hoffmann, and G. Ghosh**. 2009. The Nfkb1 and Nfkb2 proteins p105 and p100 function as the core of high-molecular-weight heterogeneous complexes. Mol Cell **34**(5): 591-602.

170. **Schaefer, L., A. Babelova, E. Kiss, H.J. Hausser, M. Baliova, M. Krzyzankova, G. Marsche, M.F. Young, D. Mihalik, M. Gotte, E. Malle, R.M. Schaefer, and H.J. Grone**. 2005. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J Clin Invest **115**(8): 2223-2233.

171. **Scheibner, K.A., M.A. Lutz, S. Boodoo, M.J. Fenton, J.D. Powell, and M.R. Horton**. 2006. Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. J Immunol **177**(2): 1272-1281.

172. **Scherer, P.E., S. Williams, M. Fogliano, G. Baldini, and H.F. Lodish**. 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. J Biol Chem **270**(45): 26746-26749.

173. **Serebrennikova, O.B., C. Tsatsanis, C. Mao, E. Gounaris, W. Ren, L.D. Siracusa, A.G. Eliopoulos, K. Khazaie, and P.N. Tsichlis**. 2012. Tpl2 ablation promotes intestinal inflammation and tumorigenesis in Apcmin mice by inhibiting IL-10 secretion and regulatory T-cell generation. Proc Natl Acad Sci U S A **109**(18): E1082-1091.

174. **Shapiro, L. and P.E. Scherer**. 1998. The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. Curr Biol **8**(6): 335-338.

175. **Soria-Castro, I., A. Krzyzanowska, M.L. Pelaez, J. Regadera, G. Ferrer, L. Montoliu, R. Rodriguez-Ramos, M. Fernandez, and S. Alemany**. 2010. Cot/tpl2 (MAP3K8) mediates myeloperoxidase activity and hypernociception following peripheral inflammation. J Biol Chem **285**(44): 33805-33815.

176. **Stafford, M.J., N.A. Morrice, M.W. Peggie, and P. Cohen**. 2006. Interleukin-1 stimulated activation of the COT catalytic subunit through the phosphorylation of Thr290 and Ser62. FEBS Lett **580**(16): 4010-4014.

177. **Sugimoto, K., M. Ohata, J. Miyoshi, H. Ishizaki, N. Tsuboi, A. Masuda, Y. Yoshikai, M. Takamoto, K. Sugane, S. Matsuo, Y. Shimada, and T. Matsuguchi**. 2004. A serine/threonine kinase, Cot/Tpl2, modulates bacterial DNA-induced IL-12 production and Th cell differentiation. J Clin Invest **114**(6): 857-866.

178. **Sun, S., T. Sursal, Y. Adibnia, C. Zhao, Y. Zheng, H. Li, L.E. Otterbein, C.J. Hauser, and K. Itagaki**. 2013. Mitochondrial DAMPs increase endothelial permeability through neutrophil dependent and independent pathways. PLoS One **8**(3): e59989.

179. **Takeda, K. and S. Akira**. 2005. Toll-like receptors in innate immunity. Int Immunol **17**(1): 1-14.

180. **Takeuchi, T., Y. Adachi, Y. Ohtsuki, and M. Furihata**. 2007. Adiponectin receptors, with special focus on the role of the third receptor, T-cadherin, in vascular disease. Med Mol Morphol **40**(3): 115-120.

181. **Taylor, P.R. and S. Gordon**. 2003. Monocyte heterogeneity and innate immunity. Immunity **19**(1): 2-4.

182. **Thakor, N. and M. Holcik**. 2012. IRES-mediated translation of cellular messenger RNA operates in eIF2alpha- independent manner during stress. Nucleic Acids Res **40**(2): 541-552.

183. **Thompson, S.R., J. Sanders, J.W. Stephens, G.J. Miller, and S.E. Humphries**. 2007. A common interleukin 18 haplotype is associated with higher body mass index in subjects with diabetes and coronary heart disease. Metabolism **56**(5): 662-669.

184. **Thoreen, C.C., L. Chantranupong, H.R. Keys, T. Wang, N.S. Gray, and D.M. Sabatini**. 2012. A unifying model for mTORC1-mediated regulation of mRNA translation. Nature **485**(7396): 109-113.

185. **Tian, L., N. Luo, X. Zhu, B.H. Chung, W.T. Garvey, and Y. Fu**. 2012. Adiponectin-AdipoR1/2-APPL1 signaling axis suppresses human foam cell formation: differential ability of AdipoR1 and AdipoR2 to regulate inflammatory cytokine responses. Atherosclerosis **221**(1): 66-75.

186. **Topisirovic, I., Y.V. Svitkin, N. Sonenberg, and A.J. Shatkin**. 2011. Cap and cap-binding proteins in the control of gene expression. Wiley Interdiscip Rev RNA **2**(2): 277-298.

187. **Tsatsanis, C., C. Patriotis, and P.N. Tsichlis**. 1998. Tpl-2 induces IL-2 expression in T-cell lines by triggering multiple signaling pathways that activate NFAT and NF-kappaB. Oncogene **17**(20): 2609-2618.

188. **Tsatsanis, C., C. Patriotis, S.E. Bear, and P.N. Tsichlis**. 1998. The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines. Proc Natl Acad Sci U S A **95**(7): 3827-3832.

189. **Tsatsanis, C., K. Vaporidi, V. Zacharioudaki, A. Androulidaki, Y. Sykulev, A.N. Margioris, and P.N. Tsichlis**. 2008. Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells. Proc Natl Acad Sci U S A **105**(8): 2987-2992.

190. **Tsuchida, A., T. Yamauchi, S. Takekawa, Y. Hada, Y. Ito, T. Maki, and T. Kadowaki**. 2005. Peroxisome proliferator-activated receptor (PPAR)alpha activation increases adiponectin receptors and reduces obesity-related inflammation in adipose tissue: comparison of activation of PPARalpha, PPARgamma, and their combination. Diabetes **54**(12): 3358-3370.

191. **Tsuchida, A., T. Yamauchi, Y. Ito, Y. Hada, T. Maki, S. Takekawa, J. Kamon, M. Kobayashi, R. Suzuki, K. Hara, N. Kubota, Y. Terauchi, P. Froguel, J. Nakae, M. Kasuga, D. Accili, K. Tobe, K. Ueki, R. Nagai, and T. Kadowaki**. 2004. Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. J Biol Chem **279**(29): 30817-30822.

192. **Turvey, S.E. and D.H. Broide**. 2010. Innate immunity. J Allergy Clin Immunol **125**(2 Suppl 2): S24-32.

193. **Unterholzner, L., S.E. Keating, M. Baran, K.A. Horan, S.B. Jensen, S. Sharma, C.M. Sirois, T. Jin, E. Latz, T.S. Xiao, K.A. Fitzgerald, S.R. Paludan, and A.G. Bowie**. 2010. IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol **11**(11): 997-1004.

194. **Vabulas, R.M., P. Ahmad-Nejad, C. da Costa, T. Miethke, C.J. Kirschning, H. Hacker, and H. Wagner**. 2001. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J Biol Chem **276**(33): 31332-31339.

195. **Vallabhapurapu, S. and M. Karin**. 2009. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol **27**: 693-733.

196. **Van Acker, G.J., G. Perides, E.R. Weiss, S. Das, P.N. Tsichlis, and M.L. Steer**. 2007. Tumor progression locus-2 is a critical regulator of pancreatic and lung inflammation during acute pancreatitis. J Biol Chem **282**(30): 22140-22149.

197. **van der Heijden, M.S. and R. Bernards**. 2010. Inhibition of the PI3K pathway: hope we can believe in? Clin Cancer Res **16**(12): 3094-3099.

198. **Van Ginderachter, J.A., K. Movahedi, G. Hassanzadeh Ghassabeh, S. Meerschaut, A. Beschin, G. Raes, and P. De Baetselier**. 2006. Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. Immunobiology **211**(6-8): 487-501.

199. **Van Huffel, S., F. Delaei, K. Heyninck, D. De Valck, and R. Beyaert**. 2001. Identification of a novel A20-binding inhibitor of nuclear factor-kappa B activation termed ABIN-2. J Biol Chem **276**(32): 30216-30223.

200. **Verhelst, K., L. Verstrepen, B. Coornaert, I. Carpentier, and R. Beyaert**. 2009. Cellular expression of A20 and ABIN-3 in response to Toll-like receptor-4 stimulation. Methods Mol Biol **517**: 205-215.

201. **Wan, Y.Y., H. Chi, M. Xie, M.D. Schneider, and R.A. Flavell**. 2006. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. Nat Immunol **7**(8): 851-858.

202. **Wang, C., L. Deng, M. Hong, G.R. Akkaraju, J. Inoue, and Z.J. Chen**. 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature **412**(6844): 346-351.

203. **Wang, X., G.Z. Feuerstein, J.L. Gu, P.G. Lysko, and T.L. Yue**. 1995. Interleukin-1 beta induces expression of adhesion molecules in human vascular smooth muscle cells and enhances adhesion of leukocytes to smooth muscle cells. Atherosclerosis **115**(1): 89-98.

204. **Wang, Y., A. Xu, C. Knight, L.Y. Xu, and G.J. Cooper**. 2002. Hydroxylation and glycosylation of the four conserved lysine residues in the collagenous domain of adiponectin. Potential role in the modulation of its insulin-sensitizing activity. J Biol Chem **277**(22): 19521-19529.

205. **Waterfield, M., W. Jin, W. Reiley, M. Zhang, and S.C. Sun**. 2004. IkappaB kinase is an essential component of the Tpl2 signaling pathway. Mol Cell Biol **24**(13): 6040-6048.

206. **Waterfield, M.R., M. Zhang, L.P. Norman, and S.C. Sun**. 2003. NF-kappaB1/p105 regulates lipopolysaccharide-stimulated MAP kinase signaling by governing the stability and function of the Tpl2 kinase. Mol Cell **11**(3): 685-694.

207. **Watford, W.T., C.C. Wang, C. Tsatsanis, L.A. Mielke, A.G. Eliopoulos, C. Daskalakis, N. Charles, S. Odom, J. Rivera, J. O'Shea, and P.N. Tsichlis**. 2010. Ablation of tumor progression locus 2 promotes a type 2 Th cell response in Ovalbumin-immunized mice. J Immunol **184**(1): 105-113.

208. **Wolf, A.M., D. Wolf, H. Rumpold, B. Enrich, and H. Tilg**. 2004. Adiponectin induces the anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes. Biochem Biophys Res Commun **323**(2): 630-635.

209. **Wulster-Radcliffe, M.C., K.M. Ajuwon, J. Wang, J.A. Christian, and M.E. Spurlock**. 2004. Adiponectin differentially regulates cytokines in porcine macrophages. Biochem Biophys Res Commun **316**(3): 924-929.

210. **Yamamoto, M., S. Sato, H. Hemmi, S. Uematsu, K. Hoshino, T. Kaisho, O. Takeuchi, K. Takeda, and S. Akira**. 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol **4**(11): 1144-1150.

211. **Yamauchi, T., J. Kamon, Y. Minokoshi, Y. Ito, H. Waki, S. Uchida, S. Yamashita, M. Noda, S. Kita, K. Ueki, K. Eto, Y. Akanuma, P. Froguel, F. Foufelle, P. Ferre, D. Carling, S. Kimura, R. Nagai, B.B. Kahn, and T. Kadowaki**. 2002. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med **8**(11): 1288-1295.

212. **Yamauchi, T., J. Kamon, H. Waki, Y. Terauchi, N. Kubota, K. Hara, Y. Mori, T. Ide, K. Murakami, N. Tsuboyama-Kasaoka, O. Ezaki, Y. Akanuma, O. Gavrilova, C. Vinson, M.L. Reitman, H. Kagechika, K. Shudo, M. Yoda, Y. Nakano, K. Tobe, R. Nagai, S. Kimura, M. Tomita, P. Froguel, and T. Kadowaki**. 2001. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med **7**(8): 941-946.

213. **Yamauchi, T., J. Kamon, Y. Ito, A. Tsuchida, T. Yokomizo, S. Kita, T. Sugiyama, M. Miyagishi, K. Hara, M. Tsunoda, K. Murakami, T. Ohteki, S. Uchida, S. Takekawa, H. Waki, N.H. Tsuno, Y. Shibata, Y. Terauchi, P. Froguel, K. Tobe, S. Koyasu, K. Taira, T. Kitamura, T. Shimizu, R. Nagai, and T. Kadowaki**. 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature **423**(6941): 762-769.

214. **Yokota, T., K. Oritani, I. Takahashi, J. Ishikawa, A. Matsuyama, N. Ouchi, S. Kihara, T. Funahashi, A.J. Tenner, Y. Tomiyama, and Y. Matsuzawa**. 2000. Adiponectin, a new member of the family of soluble defense collagens, negatively regulates the growth of myelomonocytic progenitors and the functions of macrophages. Blood **96**(5): 1723-1732.

215. **Yoshimura, A., T. Naka, and M. Kubo**. 2007. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol **7**(6): 454-465.

216. **Zhang, N., M.H. Ahsan, A.F. Purchio, and D.B. West**. 2005. Serum amyloid A-luciferase transgenic mice: response to sepsis, acute arthritis, and contact hypersensitivity and the effects of proteasome inhibition. J Immunol **174**(12): 8125-8134.

217. **Zhang, Q., M. Raoof, Y. Chen, Y. Sumi, T. Sursal, W. Junger, K. Brohi, K. Itagaki, and C.J. Hauser**. 2010. Circulating mitochondrial DAMPs cause inflammatory responses to injury. Nature **464**(7285): 104-107.